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PEOPLE AND EVENTS

Cushman Honored by Southern Dairy Science Division

In recognition of his distinguished service to the dairy industry in the South, C. G. CUSHMAN, of Clemson, has been signally honored by the Southern Division of the American Dairy Science Association.

C. G. Cushman

He received this honor at the annual awards banquet of the Southern Division at Memphis, Tenn. Feb. 3, held in connection with the annual meeting of the Southern Association of Agricultural Workers.

In recognition of his distinguished service he was presented a handsomely framed portrait of himself. Also within the frame is shown his record of achievements in the promotion of dairying in South Carolina and in other southern states during the past 38 years. The presentation was made by B. W. NEASHAM, extension dairyman, Louisiana Extension Service, and chairman of the division's honor committee.

Mr. Cushman is a native of Indiana and a dairy graduate of Purdue Univ. His dairy development work in South Carolina began in 1921 when he was named dairy specialist of the Clemson Extension Service. From 1925 to 1930 he was secretary of the Chester Chamber of Commerce and during that period helped Chester county to become one of the outstanding dairy counties of the Southeast. In 1931 he became leader of Clemson Extension Dairy Work, and has served in that capacity since, except for a few months while on "loan" to the Agricultural Adjustment Administration, Washington, as consultant in the Milk Marketing Agreement Program for the Southeastern States and as senior agricultural economist for the AAA.

During his 38-year career as a dairy leader he has worked consistently for the production and use of high quality roughages, and was a pioneer in the pasture program development in the state. He has always been firmly convinced that the dairy industry should be organized, and he has assisted in the organization of and has been closely associated with, all the dairy breed associations and producer groups in the state. To help stabilize milk prices he assisted in the organization of the state's principal milk sheds. He has played an important part in the organization and work of the artificial breeding associations in the state, and is either the author or co-author of several helpful extension dairy publications.

In 1956 he was named "Man of the Year in South Carolina Agriculture" by the Progressive Farmer Magazine. He has served as secretary and as president of the Southern Division of the American Dairy Science Association, and also as secretary of the Extension Section of the Association.

54th Annual Meeting Plans Under Way at Illinois

Plans are well under way at the Univ. of Illinois to roll out the "welcome mat" for members of the American Dairy Science Association on June 15–17, 1959, for their 54th annual meeting. Excellent facilities for general and sectional meetings and for evening entertainment programs have been made available. Space in the new modern dormitories has been reserved for guests and their families.

Pre-registration materials for the meetings will be mailed to members of A.D.S.A. Persons who are not members of the association desiring to attend the meetings may obtain pre-registration forms by writing to K. A. KENDALL, Dept. of Dairy Science, Univ. of Illinois, Urbana, Illinois.

Members of the General Steering Committee responsible for making local arrangements and plans for the sessions are G. W. SALISBURY, chairman, L. R. FRYMAN, K. E. GARDNER, E. O. HERREID, K. A. KENDALL, R. T. MILNER, W. O. NELSON, E. E. ORMISTON, MRS. G. W. SALISBURY, J. TOBIAS, R. W. TOUCHBERRY, and S. L. TUCKEY.

FDA Promotes Fischbach, Friedman

The Food and Drug Administration of the U. S. Department of Health, Education, and Welfare has announced two recent scientific appointments.

HENRY FISCHBACH will succeed O. L. KLINE as head of the Division of Food. Dr. Kline was recently named head of the FDA Division of Nutrition (see People and Events Section, January, 1959).

Dr. Fischbach received his Ph.D. in chemistry from Indiana Univ. and began his career with the FDA as an inspector at New Orleans in 1939. He has served as a research chemist in Washington from 1941-1956, when he became assistant to the director of the Bureau of Biological and Physical Sciences.

LEO FRIEDMAN has been named director of research for the Division of Nutrition. Dr. Friedman received his doctorate in biochemistry from Georgetown Univ. in 1949. Beginning his FDA career in 1939, he worked up through the ranks to become chief of the Biological Branch of the Division of Nutrition in 1950.

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First Youth Conference on Atom to Open April 30

More than 400 high school student delegates are expected to attend the First National Youth Conference on the Atom, to be held April 30–May 1 at Atlantic City, N. J.

The purpose of the conference is to present to a group of the nation's most able high school science students and teachers an authoritative and inspiring picture of the peaceful atom in all of its various applications. The program is also designed to help build interest in the study of science in the U.S.

The program is being arranged by the Electric Companies Public Information Program, New York City. This organization is a national public relations organization for the electric utility industry.

Program speakers include J. A. McCone, chairman of the Atomic Energy Commission; C. E. Robbins, executive manager of the Atomic Industrial Forum; Cyril Comar, director, Laboratory of Radiation Biology, Cornell Univ.; John Laughlin, division of physics, Sloan-Kettering Institute for Cancer Research; C. H. Weaver, vice president of Westinghouse; F. K. McCune, vice president of General Electric; and N. C. Hilberry, director of Argonne National Laboratory.

Cooperating organizations are American Association for the Advancement of Science, AEC, Atomic Industrial Forum, National Science Foundation, National Science Teachers Association, Science Clubs of America, and Future Scientists of America Foundation.

Louisiana A.D.S.A. Division To Meet April 17

The Annual Meeting of the Louisiana Division of A.D.S.A. will be held April 17 at Southeastern Louisiana College, Hammond.

High lights of the program are (1) a film entitled "This Is Your Dairy Industry," (2) a report on an interpretation of research relating to SNF and freezing point of milk, (3) evaluation and recommendations of sanitary standards for milk produced in Louisiana, (4) a panel on "Problems Facing the Dairy Farmer and Plant Operator in Louisiana," and (5) a talk by A. C. Fay, vice-president of A.D.S.A.

After the meeting, farm tours will be conducted under the direction of B. J. Burch. Reservations for the meeting may be made at the Hotel Casa De Fresa, Hammond.

Recent Death

O. A. Jamison, 69, market administrator for the Federal Milk Order, San Antonio, Tex., died July 18, 1958.

Mr. Jamison received his B.S. from Ohio State Univ. in 1912 and his M.S. in 1916 from the Univ. of Maine. He also took further graduate work at the Univ. of Massachusetts.

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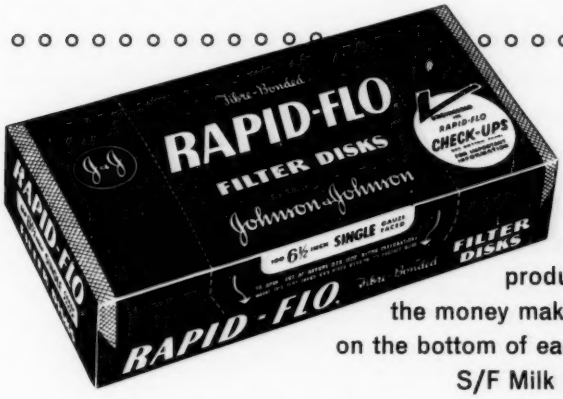

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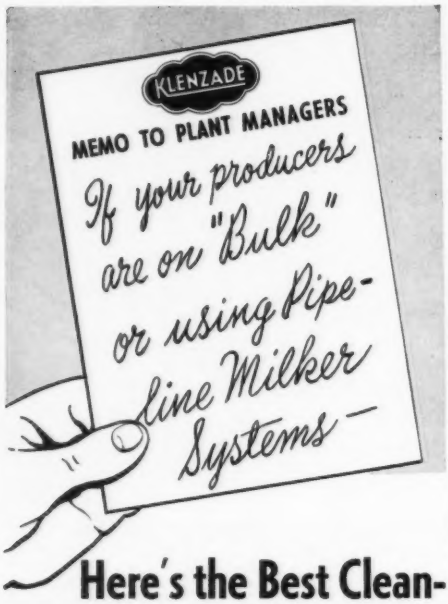

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He began his agricultural career as a teacher at Michigan State Univ. in 1912, and in 1934 became a marketing specialist for the USDA. After holding government posts throughout the country, he became a marketing specialist in Washington in 1941. In 1954 he was named to his post in San Antonio.

Mr. Jamison is survived by his widow; a son, W. R. JAMISON, Burbank, Calif., and a daughter, MRS. DORETHEA LARA, Glendale, Calif.

Int. Dairy Congress to Open with Royal Welcome

An estimated 3,000 visitors at the 25th International Dairy Congress, to be held in London, England, June 29–July 3, will receive a royal welcome at the opening session June 29.

Queen Elizabeth and the Queen Mother will open the meeting at Royal Albert Hall, and the proceedings will be distinguished by all of the colorful ceremonial of an historic occasion. A full band of the Scotts Guards—including pipes and drums—and the Trumpeters of Kneller Hall will be on hand for the event.

Sir THOMAS PEACOCK, C.B.E., who is chairman of the United Kingdom Dairy Association as well as of the Congress, will welcome the visitors. PAUL KASTLI, president of the International Dairy Federation, will also speak.

During the two-week event, parties of Congress members will tour Britain to see farms, creameries, dairy institutes, and factories, as well as historic towns.

Completed Theses

M.S. Degree

D. D. COLE—Gas-liquid chromatographic analysis of ammonia and volatile amines and their relationship to certain flavor defects in milk. Ohio State Univ., Columbus.

FRANK KOVAL—The effect of modern dairy industry practices on the free fatty acid content of milk. Ohio State Univ., Columbus.

H. E. RANDOLPH—The suitability of low-heat nonfat powder for the manufacture of cottage cheese. Ohio State Univ., Columbus.

Ph.D. Degree

R. C. DUTRA—The nature and origin of some flavor components of evaporated milk. Univ. of California, Davis.

Shaw Leaves Maryland

J. C. SHAW, Professor of Dairy Husbandry at the Univ. of Maryland, has resigned his position to become Chief of the Animal Production Branch, Animal Production and Health Division of the Food and Agriculture Organization of the United Nations in Rome, Italy. He assumed this position the first of the year.

DAIRY TOURS OF EUROPE

in connection with the

XV International Dairy Congress, London June 29-July 3

European Traveling Seminar offers post-congress dairy excursions of England, Denmark, Sweden, Holland, France, Germany, Switzerland, Italy. There will be visits to dairy plants, including the famous French, Swiss and Italian cheese producers, farms, plants for the manufacture of dairy equipment. Talks with dairy scientists. Visits to laboratories. Stays with farm families. Groups of 15 American dairy participants under leadership of American dairy scientists. Special programs will be arranged for wives. All this combined with regular sightseeing. Extension tours may be arranged to Norway, Finland, Ireland, Scotland, Austria, Spain, Portugal, etc.

ETS, in its 11th year of operation, specializes in scientific and professional study tours, and has excellent contacts with European scientists, governments, dairy farmers, dairy industry. These tours will be prepared in cooperation with Agricultural Departments of European governments.

ETS makes all arrangements for transatlantic passage, hotels, meals, transportation in Europe, sightseeing, etc. These arrangements are inexpensive because all facilities handled directly and locally . . . no middle-men.

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Ohio State News

The 42nd Annual Convention of the Ohio Dairy Products Association was held in January at Cincinnati.

R. E. WEBNER, general manager of the Cottage Creamery Co., Orrville, was named president of the association for 1959. JOHN BURNS, district manager, Beatrice Foods, Inc., was re-elected treasurer.

The Eighth Annual Short Course in Milk Sanitation and Public Health was held in March at Ohio State Univ., Columbus. The event featured "Plant and Farm Cleaning and Sanitizing."

The event was sponsored by the Dept. of Dairy Technology in cooperation with the Ohio State Dept. of Agriculture and Health. Milk sanitarians and industry people attended the course.

L. to R.: Wayne Babcock, Babcock Dairy, Toledo; Leo Soehnlen, Superior Dairy, Canton; T. Kristoffersen, Ohio State University; Robert Hamilton, Willar Dairy, Willard; Harold Reiter, Reiter-Harter Dairy, Akron; Fred Campbell, Andulusia Dairy, Salem; M. Peeples, Ohio State University; D. Charles, Meyer Dairy Products Co., Cleveland; Robert Carr, Dairymen's Producers Co., Cleveland; L. Kesselring, Kesselring Dairy, Akron; F. Greiner, Exec. Secy., Ohio Dairy Products Association, Columbus; I. A. Gould, Ohio State University; Carl Broughton, Broughton's Farm Dairy, Marietta; William Deeds, Deeds Bros. Dairy, Lancaster; F. Grieser, Napoleon Creamery, Napoleon; A. L. Shough, Kroger Co., Cincinnati; guest of Mr. Shough; Irving Reynolds, Franklin Ice Cream Co., Toledo.

The Australian Journal Of Dairy Technology

The Journal contains scientific and technical papers on all aspects of dairying, especially dairy manufacture. It provides for those in other countries an up-to-the minute record of the latest developments in dairy technology in Australia and New Zealand. News, reports, editorials, book reviews and Society proceedings are included, and the Journal is illustrated. It appears quarterly.

SOME ARTICLES PUBLISHED IN 1957-58.

"Progress in the Engineering of Dairy Processing" by J. K. Scott.

"Mechanization of Milling, Salting and Hooping in Cheddar Cheese Manufacture" by J. Czulak, N. H. Freeman and H. L. C. Chapman.

"The Stability of Vitamin A in Reconstituted Fortified Non-Fat Milk Solids, Part I—The Effect of Heat; Part II—The Effect of Light"—by J. Conochie and R. A. Wilkinson.

"Modern Packaging of Cheese" by W. J. Park.

"The Effect of Temperature on the Keeping Quality of Butter in Cold Storage" by E. G. Pont and L. F. Gunnis.

"Variations in Lactic Acid Production in Milk" by Barbara P. Keogh.

Obtainable through booksellers, or from the Australian Society of Dairy Technology, P.O. Box 20, Highett, Victoria, Australia. Price $3.50 per annum.

Special Offer: **A certain number of complete sets of back numbers, comprising twelve volumes and a decennial index, are available at a concession price of $27.00.**

New division chairmen included ED MOSER, Med-O-Pure Dairy, Washington C. H.; RICHARD BEUHLER, M&R Dietetic Laboratory, Columbus; ROGER UMBS, San-A-Pure Dairy, Findlay; and HAROLD HAMILTON, Eldorado Creamery, Eldorado.

Mr. Webner named an education committee for the association which will work with the Dept. of Dairy Technology in respect to the education and manpower problems. CARL BROUGHTON, Marietta, is chairman of the committee.

To acquaint the business leaders of Ohio's dairy industry with the programs and problems in education, research, and service, the Dept. of Dairy Technology held four meetings in December and January. The discussion on education encompassed the points of (1) supply of young talent, (2) the curriculum, and (3) post graduation development of leaders. The role of industry in meeting the cost of expanding research activities and the needs for on-campus and off-campus adult educational programs also came up for discussion.

FRANK KOVAL, extension specialist in the department since 1954, has resigned to become executive secretary of the Michigan Dairy Products Association.

J. J. BETSCHER has resigned as instructor in the department to devote full time to graduate study.

J. G. KAPSALIS has been appointed research assistant in the department. Dr. Kapsalis is a native of Greece where he received his B.S. degree in agriculture from Athens College. He recently received his Ph.D. degree from Texas A&M. He will do research on the spreadability of butter.

Dairy Society International Names Phillipsen Organization Director

Following a recent Board of Directors meeting, in Boston, Dairy Society International's managing director ROBERTS EVERETT announced the appointment of W. L. PHILLIPSEN to a new post of Organization Director. Phillipsen, 38, is former general manager of the American Dairy Association of Wisconsin.

Mr. Phillipsen has had long experience in the dairy industry. He was born on a Wisconsin dairy farm, and was graduated with a B.S. degree in Agriculture from the Univ. of Wisconsin. He served in his home state as a high school Agriculture teacher, in Cumberland, and as Agricultural Extension Agent in Dunn County before becoming associated with the American Dairy Association in 1952. As manager of the Wisconsin ADA, he was responsible for state advertising, merchandising, public relations, and membership activities. The Wisconsin unit is the largest state unit in the

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Food intake is largely controlled by our body's demand for energy. Wise selection of food is necessary to insure that we satisfy nutrient needs while we satisfy energy demands.

Foods combined in the minimum amounts suggested in *A Guide to Good Eating* provide most of the nutrient needs and about 2/3 the energy needs of the average healthy active adult. Of the adult Recommended Dietary Allowance, these amounts of

milk and dairy foods supply about 1/7 of the calories . . . foods in the meat group supply about 1/5 of the calories . . . vegetables and fruits supply about 1/9 of the calories . . . breads and cereals supply about 1/8 of the calories.

More of these or other foods . . . with moderate use of sugars and syrups, fats and oils in food preparation and at the table . . . quickly increase the calorie intake to meet energy needs. An adequate supply of energy is essential if the body is to make efficient use of dietary protein.

A GUIDE TO GOOD EATING — USE DAILY

DAIRY FOODS

3 to 4 glasses milk—children • 4 or more glasses—teenagers • 2 or more glasses—adults • Cheese, ice cream and other milk-made foods can supply part of the milk

MEAT GROUP

2 or more servings • Meats, fish, poultry, eggs, or cheese—with dry beans, peas, nuts as alternates

VEGETABLES AND FRUITS

4 or more servings • Include dark green or yellow vegetables; citrus fruit or tomatoes

BREADS AND CEREALS

4 or more servings • Enriched or whole-grain added milk improves nutritional values

Thus, even in reducing diets, calories from carbohydrates and fats should be included.

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The nutritional statements made in this advertisement have been reviewed by the Council on Foods and Nutrition of the American Medical Association and found consistent with current authoritative medical opinion.

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American Dairy Association, furnishing approximately 20 per cent of the national ADA budget.

DSI's Board also designated G. W. WEIGOLD of Torrington, Conn., and Washington, D.C., as Projects and Technical Director for the international Society. He will be closely concerned with the overseas projects in which the Society cooperates with the Foreign Agricultural Service of the USDA.

Cornell News

FRANK KOSIKOWSKI of the Dept. of Dairy Industry has accepted an invitation of the U. S. State Dept. to participate in a new scholarship exchange program in Ireland.

This spring, he will engage in research and discussions pertaining to his field with Irish scientists. During his stay abroad, he will be working in the laboratory of G. T. PYNE, Univ. College, Cork, Ireland.

The following schools will be held at Cornell this spring: Refrigeration Training School, April 1-2; Detergent Training School, April 15-16, and Grade II Milk Inspectors Training School, May 11-15 and May 18-22.

Further details about these schools may be obtained from J. C. WHITE, Dept. of Dairy Industry, Cornell Univ., Ithaca, N. Y.

Connecticut Holds Short Courses

The Univ. of Connecticut recently held two short courses—Vacuum Flavor Removal Equipment Operation and Soft-Serve and Soda Fountain Operation.

The first course was attended by 40 plant men, inspectors, and sales engineers from Massachusetts, Rhode Island, New York, and Connecticut. A study was made of the proper method of sanitizing, operating, and in-place-cleaning the creamery's vacuum equipment, followed by inspection of the various safety features of the vacuum equipment accessories.

Twenty soft-serve operators from throughout the state attended the latter course. The subject matter covered included ice cream, ice milk, and milkshake mix processing and selection, care and operation of soft-serve freezers and milkshake machines, fountain operation, sanitation and housekeeping, merchandising, record keeping and cost analysis. Guest speakers were MRS. D. H. MACKENZIE, vice-president of the National Association of Retail Ice Cream Manufacturers, Keene, N. H.; ARTHUR CARDINAL, H. A. Johnson Company, Boston, Mass.; and E. E. SMITH, Food and Drug Commission, Hartford, Conn.

Future short courses include an Ultra-High Temperature Pasteurization Conference on April 14, 1959. Individuals interested in the complete program may write to A. I. MANN, Director, Rateliffe Hicks School of Agriculture, Univ. of Connecticut, Storrs.

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Arizona News

The Eighth Annual Dairy Industry Conference was held in January at the Univ. of Arizona, Tueson.

Participants on the program included J. A. BANKS, vice-president, Associated Dairy Products Co., Glendale, Ariz.; R. A. HARVILL, president, Univ. of Arizona; J. L. ALM, Safeway Stores, Inc., Los Angeles, Calif.; P. H. TRACY, Dept. of Food Technology, Univ. of Illinois, Urbana; M. H. LOHMAN, general manager, Arden Farms Co., Phoenix; K. W. MICHAEL, manager, Community Relations and Communications, Computer Dept., General Electric, Phoenix; D. R. MOORE, vice president, Capital Business Service, Lansing, Mich.; GOVERNOR KEEN JOHNSON, Reynolds Metals Co., Louisville, Ky.; BRUCE KNAPP, president, Sunset Dairy, Tueson; R. A. SIMONET, manager of

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field service, Robert A. Johnson Co., Milwaukee, Wis.; P. S. KENMOEN, work simplification coordinator, Carnation Co., Los Angeles, Calif.; W. V. JOHNSON, plant manager, Safeway Stores, Phoenix; J. S. CUNNINGHAM, executive assistant, Dairy Industries Supply Association, Washington, D.C., and J. C. PFEFFER, Safeway Stores, Inc., Oakland, Calif.

Foreign Dairy Market Drops

Foreign market prospects for U. S. Dairy products exports were poorer than they were last year at this time, according to a January report of the Foreign Agricultural Service. The report also stated that longer-run considerations do not promise much improvement.

Among the reasons given was that milk production is increasing faster than population growth in the principal dairy producing and consuming areas. Per capita consumption of dairy products is increasing very slowly or is virtually static in some important countries.

The net result, according to the FOA report, is larger available supplies of exportable dairy products and shrinking import markets.

H. W. Wiley Award Nominations Close

Nominations are now in for the third Harvey W. Wiley Award for Achievement in Analytical Methods, a scientific award sponsored annually by the Association of Official Agricultural Chemists.

The award, which consists of $500 in cash, goes to the scientist who makes any outstanding contribution to the development of methods of analysis for foods, drugs, cosmetics, feeds, fertilizers, and pesticides, or for use in general analytical chemistry.

These are the fields of interest of the AOAC encompassed in its publication, *Official Methods of Analysis*. This book is the primary laboratory manual of regulatory chemists and research workers in agriculture in the U. S. and other nations.

MIF Sales Course Graduates

The January Milk Industry Foundation Sales Training Institute, which was completed recently, brought the total number of graduates to 1,218 over the past eight years.

January graduates were ED CZECZOK, Marigold Dairy, Winona, Minn.; H. P. GARDNER, Dairymen's League Cooperative Association, Poughkeepsie, N. Y.; J. K. HOFFMAN, Bancroft Dairy Co., Madison, Wis.; ROBERT KOLBACK, Marigold Dairies, Mason City, Ia.; W. W. MOSLEY, JR., Carnation Co., Tulsa, Okla.; WARREN PARKER, Farner & Parker, Inc., Gowanda, N. Y.; R. W. POPP, Diamond Milk Products, Columbus, O.; N. C. RUTHERFORD, Himes Bros. Dairy, Dayton, O., and C. R. VANCE, Marigold Dairies, Inc., Red Wing, Minn.

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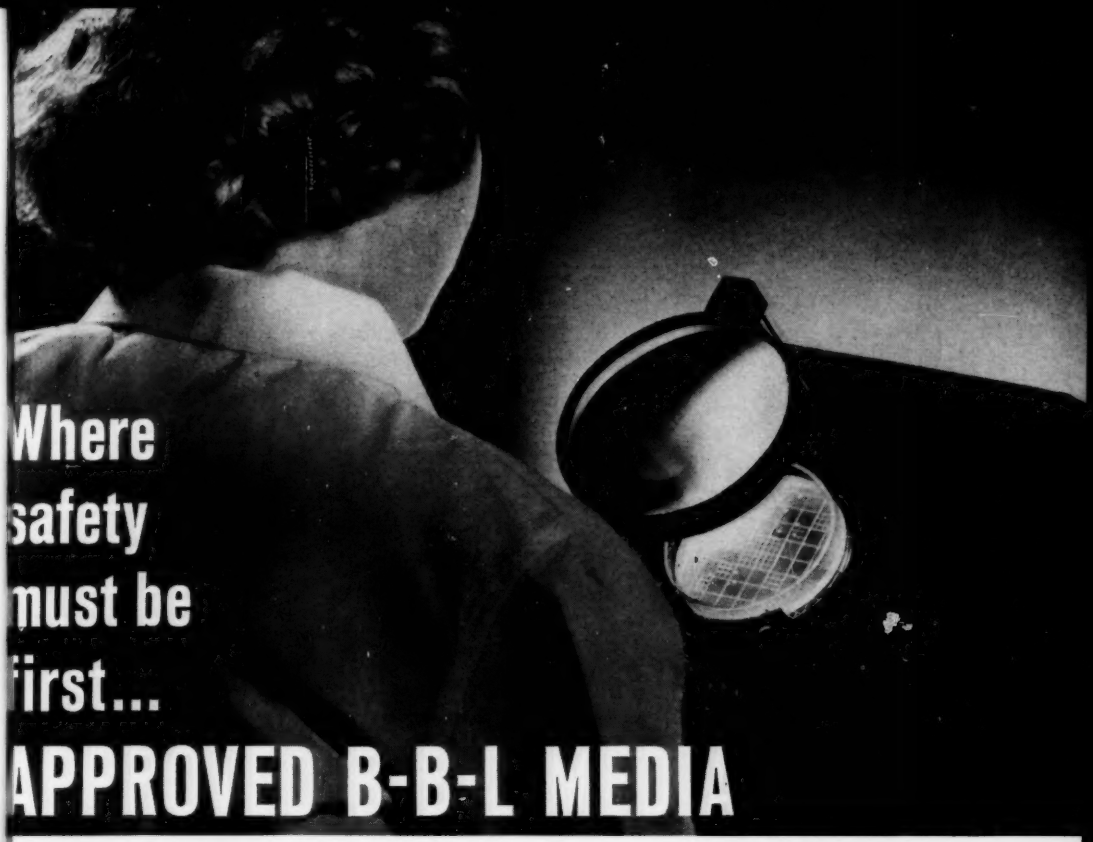

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-B-L 01-286—**VIOLET RED BILE AGAR**

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) Standard Methods for the Examination of Dairy Products, ed. 10, New York, American Public Health Association, 1953. (2) Committee Report, m. J. Pub. Health **42**:1131 (Sept.) 1952. (3) Microbiological Methods, report at 66th Ann. Meet. Assn. Official Agricultural Chemists, Sept. 29, 1952; Assn. Official Agr. Chem. **36**:91 (Feb.) 1953. (4) Standard Methods for Examination of Water, Sewage and Industrial Wastes, ed. 10, New York, merican Public Health Association, 1955.

iterature on B-B-L's **complete** line of culture media and apparatus for the microbiological laboratory sent on request. (13)

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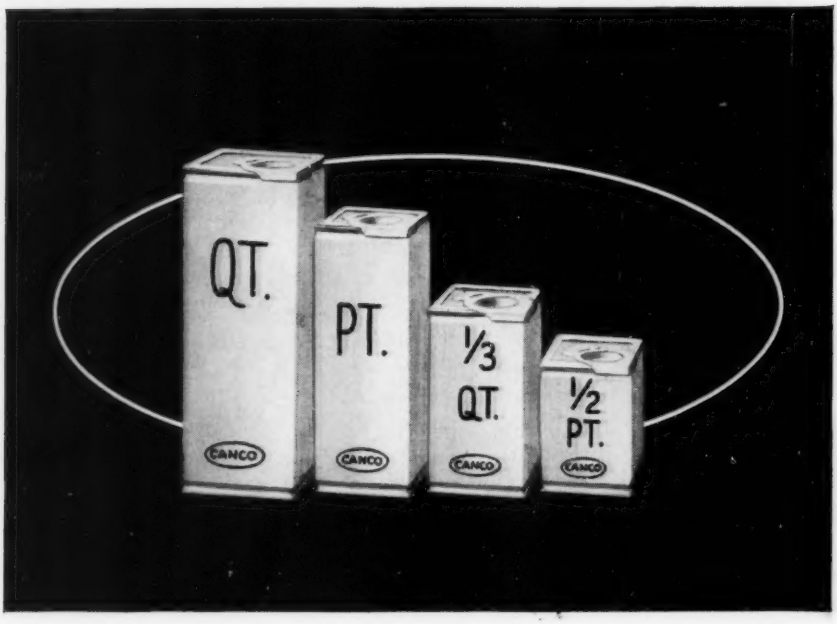

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DAIRY TECHNOLOGY SOCIETIES

Kansas City Area—JACK SMITH, chief engineer, Meyer-Blanke Co., Kansas City, was the speaker at the January meeting. His topic was "New Dairy Manufacturing Equipment."

Ohio Dairy Tech Societies—N. C. ANGEVINE, Meyer-Blanke Co., St. Louis, was speaker for the January meetings of the four Ohio societies —Maumee Valley, Northeastern, Central Ohio, and Cincinnati. His topic was "Timely Tips on the Production and Merchandizing of Quality Cottage Cheese."

Atlanta—"Controlling Flavors in Milk," was the topic discussed at the February meeting. W. M. ROBERTS, head of the dairy manufacturing section, North Carolina State College, Raleigh, was the main speaker.

Michiana—D. R. FRAZEUR, Purdue Univ., Lafayette, Ind., spoke at the February meeting on the topic, "Outlook for Dairy Industry."

Philadelphia—H. E. CALBERT, Univ. of Wisconsin, spoke at the February meeting on the topic, "The Status of Concentrated Milk."

Central Illinois—Bloomington was host to the Central Illinois Society. Featured speaker was C. A. ABELE, director of public health research, The Diversey Corp. His topic was "Corrosion of Stainless Steel."

North Carolina—North Carolina Dairy tech men heard and saw a travelogue of the dairy industry in Europe and Russia at their February meeting. The traveler was HOWARD GOFORTH of Lexington, N. C.

Detroit—The February meeting for Detroiters featured O. W. KAUFMANN, Dept. of Microbiology and Public Health, Michigan State Univ. His topic was "High Temperature Pasteurization of Ice Cream and Milk."

Metropolitan—New Yorkers heard A. E. ABRAHAMSON, chief, Wholesale Food Establishment Inspections, New York City Department of Health. He spoke on the topic, "Bacteriologic Results on Prepared Frozen Foods." The nominating committee also submitted a slate of officers including F. R. KRONENWETT for president, JOHN GRATTAN for vice president, S. A. LEAR for sergeant-at-arms, and BERNARD TZALL and ALFRED AHRENS for secretary-treasurer.

Minnesota—C. F. WEINREICH, research manager, Cherry-Burrell, Cedar Rapids, Ia., spoke at the February meeting on "Future Planning, New Equipment, New Products."

Tri-State—The Tri-State Society members met in Pittsburgh for their February meeting. R. R. KOUNTZ, Dept. of Sanitary Engineering, Pennsylvania State Univ., spoke at this meeting.

Tri-Cities—D. C. KING, head of the Dept. of Physiology, Purdue Univ., Lafayette, Ind., spoke at the February meeting. His topic was "Factors in Supervisory Leadership."

Central Michigan—In addition to installing 1959 officers, the group heard a talk in February by G. G. RIPMA, Cherry-Burrell Co., Cedar Rapids, Ia. Mr. Ripma spoke about conveyor and materials handling.

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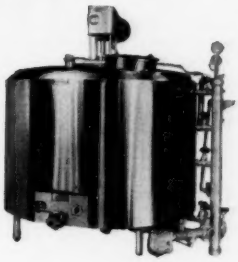

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FROM THE SECRETARY'S DESK

L. K. CROWE, associate chief adviser of the Univ. of Nebraska faculty in Turkey, has written at length about his early impressions of Turkey. He has been there since October, 1958, under the sponsorship of the U. S. Operations Mission in Turkey.

Portions of his letter follow:

"I was granted a leave of absence and accepted an appointment by the University of Nebraska for two years in Turkey to assist in the establishment of the new Ataturk University at Erzurum, Turkey. This University is to be patterned after our Land-Grant type of universities and of course this represents quite a change from the usual concept of a Turkish university.

"Mrs. Crowe and I arrived in Turkey on October 25, spent three weeks in Ankara and then came to Erzurum. The new University officially opened its doors on November 17 in a girls'-school building which had been remodeled. We have 170 students, of whom 11 are girls. We have two faculties, or colleges, Agriculture and Letters and Science. Courses are being taught this year in Mathematics, History, English, German, French, Turkish, Economics and Animal Husbandry. A beautiful building site outside of Erzurum with a background of mountains has been selected. The only evidence of the University-to-be on this site at present is some excavations.

"Ox carts, donkeys with heavy loads on their backs, and taxis which consist of two horses hitched to a buggy, are common sights on the street in front of our present building.

"My duties and responsibilities here are represented to some extent by the enclosed card. The major part of my duties are advisory in attempting to establish this new University but I am also doing some teaching. I find myself doing some advisory work with students and hope to have the opportunity of enlarging on this phase of my work. At present the language barrier makes it difficult. We expect to return to Nebraska at the end of two years where I will resume my regular duties.

"The dairy industry in this area is practically nil with the exception of some cheese manufactured from cow's, goat's and sheep milk in the spring of the year. We have every reason to believe that we can render a service in assisting this industry."

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STUDENT CHAPTER NEWS

Colorado State University

The dairy club had a very active year during 1957-58. Early in the fall of 1957 the faculty sponsored its annual student-faculty banquet. Next the club participated in Activities Night for freshmen and also won a trophy for best display at the Little National Western Stock Show. Cattle and products judging teams were sponsored by the club during this fall quarter which also saw the beginning of an annual project; the preparation and selling of a Christmas cheese box.

Winter quarter was fairly quiet for the club. An industry-student banquet started the quarter's activities. The club decided to venture again and plans were drawn up for a yearbook. Final project of winter quarter was its sponsorship of an intracollegiate judging contest.

Spring quarter was a busy one for dairy club and plans for selling concessions at College Days started the quarter. The editorial staff finished the yearbook and sent it over the state and to some neighboring Universities and Colleges. A Guernsey heifer purchased a year ago by the club was sold this quarter and topped the Northern Colorado State Guernsey Sale. The club's annual picnic finished our school year of 1957-1958.

As in the past, the faculty student banquet started Dairy Club activities during the 1958 fall quarter. At Activities Night the Dairy Club was the most popular club on campus as everyone enjoyed the free ice cream we distributed. The club won second in its display at the Little National Western Stock Show. The club again sponsored a dairy cattle judging team which traveled to Kansas City and Waterloo. To conclude fall quarter, the Dairy Club prepared and sold Christmas cheese boxes.

Texas Tech

The Dairy Industry Club of Texas Technological College has just completed a very successful fall semester here at Texas Tech. The Dairy Industry Club, consisting of 35 members, has participated in such activities as building a homecoming display for the 29th annual Tech Homecoming which was held in November, and preparing a homecoming breakfast for Alumni of the Department of Dairy Industry, school officials, and the present Dairy Industry Club members. The annual Christmas party was held in December.

The Dairy Industry Club has scheduled for its spring activities such events as the annual spring Dairy Industry Club Banquet. The members of the Dairy Products Judging Team who judged in the Southern Dairy Products Judging Contest held in Knoxville, Tennessee and the Collegiate Student's International Contest in judging Dairy Products held at Chicago, Illinois, will be presented medals. The team was coached by Dr. J. J. Wilingham, Head, Department of Dairy Industry.

The Dairy Industry Club will have a softball team to participate in the college intramural softball activities coached by E. R. Jarman instructor in Dairy Industry and sponsor of the Dairy Industry Club.

The Club will also have a concession stand to sell milk, chocolate drink, and ice cream to the people participating in the Regional F.F.A. Judging Contest this spring on the Texas Tech Campus.

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APPARENT SPECIFIC VOLUME OF THE CALCIUM CASEINATE–CALCIUM PHOSPHATE COMPLEX IN MILK

T. F. FORD,[1] G. A. RAMSDELL,[2] AND T. G. ALEXANDER[3]
Eastern Utilization Research and Development Division, USDA, Washington, D.C.

SUMMARY

Skimmilks were progressively depleted of casein colloids by centrifuging, supernatant liquids and deposited colloids being thus obtained. The deposits were washed by redispersing in distilled water and recentrifuging. Densities were determined on the supernatant liquids and on water suspensions of the washed colloids. The analysis of the data on supernatant liquids is novel, in that the apparent specific volume is first calculated for the casein complex hydrate and the specific volume of the unhydrated complex derived from this, a procedure required because the solvent is not water but milk serum. Within close limits, the values obtained are the same as those calculated directly from the densities of suspensions of washed deposits, and also agree with values calculated from the separate specific volumes of the components of the complex. The apparent specific volumes found vary slightly with composition, and are to a first approximation linearly related to the calcium content of the complex. For a colloid fraction of milk of average calcium content the apparent specific volume found is 0.697, and the extremes are 0.694 and 0.700, all with probable errors of about 0.4%. Use of these values in calculating the densities of the hydrated and solvated casein colloid particles as they exist in milk is discussed. The data are for Jersey and Holstein milks.

Data presented in previous papers (9, 10) show that the casein colloid in milk, in the broad intermediate range of sizes, contains a phosphoprotein which has the same phosphorus: nitrogen ratio in all these sizes. Similar data have since been obtained for the very smallest particles (7), and give the same ratio. There is evidence that the extremely large particles may differ in phosphoprotein composition (9), but to what extent this apparent difference may be due to inclusion of foreign matter such as leucocytes is unknown. In some skimmilks the large particles may represent as much as 15% of the total casein; in other milks they may be entirely absent.

It has also been shown that the casein colloid particles as they exist in milk, i.e., in their native state, sediment in the ultracentrifuge with a limited number of definite sedimentation velocities (6, 7). This observation, combined with the constancy of phosphoprotein composition, strongly suggests that all of these particles are aggregates of a single-unit particle.

In raw milk native casein, the phosphoprotein above discussed is intimately associated with calcium and inorganic phosphorus, in such stoichiometric proportions as to indicate a calcium caseinate–calcium phosphate complex with some substitution of magnesium for calcium (1, 9, 13). The dispersed casein colloid particles also contain hydrate water (4) and considerable milk serum which is present as interstitial or solvate liquid (5).

Received for publication August 8, 1957.

[1] Present address: U. S. Naval Research Laboratory, Washington, D. C.
[2] Present address: Venice, Florida.
[3] Present address: Food and Drug Administration, U.S. Dept. of Health, Education and Welfare, Washington, D. C.

This paper has to do first with the apparent specific volume—the reciprocal of the apparent density—of the complex; second, with the specific volumes and densities of the actual hydrated and solvated particles.

Although the phosphoprotein contained in the particles is apparently of definite composition, and the proportion of calcium as calcium caseinate may also be considered to be definite, the proportion of calcium phosphate is variable over the particle-size range and between milks. The limits of this variability have been established (9, 10). It is necessary, similarly, to establish a median value of the apparent specific volume, and to set probable limits of deviation from this mean value. Although the deviations turn out to be relatively small, they must be considered in careful calculations of particle sizes from sedimentation velocity data, and in other calculations.

Apparently, only one other measurement of the specific volume of the casein complex, formerly called calcium caseinate, has been reported. This is by Nichols *et al.* (12) and is in error. A recalculated value, given in the fourth section of this paper, is in good agreement with the present results.

EXPERIMENTS AND CALCULATIONS

Values Based on Density, Total Nitrogen, and Casein Nitrogen Determinations, on Skimmilk Samples Progressively Depleted of Casein Colloids by Centrifuging. Figure 1 is a typical plot showing the relationship between density and total nitrogen in liquid samples as the casein-containing colloids are pro-

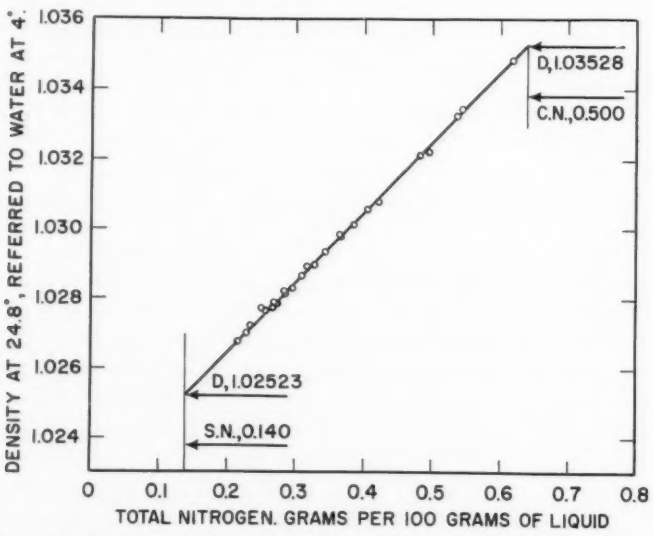

FIG. 1. Relationship of density: total nitrogen for skimmilk samples centrifuged for different lengths of time. (S.N. = serum nitrogen; C.N. = casein nitrogen. Densities measured at 24.8° and referred to water at 4°.)

gressively removed by centrifuging. Each point gives the density and nitrogen content of the liquid phase after a definite time of centrifuging. The serum nitrogen value shown was obtained by extrapolating a casein nitrogen : total nitrogen plot to zero casein nitrogen. The slope of the casein nitrogen plot, i.e., the ratio of casein nitrogen removed to total nitrogen removed is 1.00, which is the reason 0.50% casein nitrogen falls at 0.640% total nitrogen in this case.

The least-squares straight line is drawn through the set of points in Figure 1. Six such plots were obtained, for six milks, and in only one case was there any indication of curvature. This curvature, if real, is very slight. Some downward curvature should exist because of differences in composition over the size range, but it would be difficult to detect. The approximate linearity of such plots justifies only calculation of single average values for the specific volume for all of the particles in the size ranges represented.

The apparent specific volumes were calculated by the equation of Svedberg and Chirnoaga (15).

$$V = \frac{\omega - (l - h)}{\rho h}, \qquad (1)$$

where ω is the weight of serum or solvent that can be contained in the pycnometer, l is the weight of solution contained by the pycnometer, h is the weight of solute in the solution, and ρ is the density of the serum or solvent.

In calculations based on data such as those of Figure 1, it is convenient to choose a liquid sample containing, say, 0.5% casein nitrogen, and to consider this to be the solution. Assuming an average complex : nitrogen factor of 6.89 (10) and a hydration of 0.52 g. of hydrate water per gram of dry complex (4, 10), such a solution contains (0.5) (6.89) (1.52) or 5.236 g. of hydrated complex. The hydrated complex is considered to be the solute. The solvate liquid associated with the hydrated complex is by definition the same as the serum or solvent and is considered to be a part of the solvent. Referring to Figure 1, as an example, and assuming a 100-ml. pycnometer, since the density of the solution at 0.5% casein nitrogen (0.640% total nitrogen in this case) is 1.03528, the value to be used for h in Equation (1) is (5.236) (103.528)/(100) or 5.241, and $l = 103.528$. The hypothetical 0.5% casein nitrogen skimmilk contains 0.140 g. of serum nitrogen per 100 g., as does the casein-free serum, but this serum nitrogen of the skimmilk is all in the serum phase. The true concentration of serum nitrogen in the uncentrifuged serum or solvent is, therefore $\{100/(100 - 5.236)\}$ $\{0.140\}$, or 0.148 g. per 100 g. As an approximation, the density of such a serum may be taken from the plot at 0.148% total nitrogen. This makes $\rho = 1.02541$ and $\omega = 102.541$. The serum density calculated by adding to 100 g. and the corresponding volume of centrifuged casein-free serum that weight and volume of serum protein centrifugally removed with 5.236 g. of hydrated complex is 1.02540, or practically the same as found by the simple approximation. The effect of the small amount of lipid material removed is negligible. Putting the above numerical values for ω, l, h, and ρ in Equation (1) gives $V_{\text{hydrate}} = 0.798$.

If the partial specific volumes of the dry complex and hydrate water are

additive, then

$$V_{\text{complex}} = (l + y)\, V_{\text{hydrate}} - {}^{y}V_{H_2O}, \qquad (2)$$

where y is the hydration in grams per gram. Making $V_{\text{hydrate}} = 0.798$ as found above, $y = 0.52$, and as a first approximation $V_{H_2O} = 1.003$, the reciprocal of the density of water in bulk at 25°, this equation gives $V_{\text{complex}} = 0.692$ for this particular milk.

The assumption that the density of hydrate water is the same as the density of water in bulk is arbitrary and may not be justified. It is the usual assumption made in such calculations. More refined interpretation of such data must wait on better understanding of the nature of hydrate water.

Table 1 gives the values for the apparent specific volume, calculated as described above, for the six skimmilks for which density data were obtained. In Table 1 the corresponding calucium : casein nitrogen and complex : casein nitrogen ratios also are given, together with the serum nitrogen and the density values used in the calculations. Of these experiments, those for October, 1946, and for August, 1955, have been previously reported in part (*cf.* References 9 and 10). Figure 1 is a plot of the data for December, 1946. Fewer points were obtained in the last three experiments, but with greater accuracy, over wider particle-size ranges, and the plots show even less deviation from linearity.

Values Based on Density and Total Solids Determinations on Skimmilks and Their Casein-Free Serums. If only total solids and density are known for a skimmilk and its casein-free serum, an apparent specific volume can be calculated which is to a close approximation the apparent specific volume of the complex. In the October, 1946, and the two August, 1955, experiments (Table 1), total solids as well as densities and nitrogens were determined. The data are given in the preceding paper (10). Taking the August, 1955, experiment on Jersey milk as an example, the casein-free serum total solids is 6.29%, and the total solids at 0.5% casein nitrogen is 9.40%. If the amount of total solids removed from 100 g. of this skimmilk is x and if a hydration of 0.52 g. per gram of dry complex be assumed, then $9.40 - 6.29\{(100 - 1.52\ x/100\} = x$, and $x = 3.44$. Since the density of this skimmilk is 1.03300, $h = (3.44)\ (1.52)\ (1.03300)$, or 5.40. The serum density is 1.02330. Putting these values in Equation (1) gives $V_{\text{hydrate}} = 0.802$, and $V_{\text{complex}} = 0.697$. In this case, correction of the serum total solids for the serum protein and fat or lipids removed by centrifuging, and correction of the serum density for the serum protein removed, is possible. The effect of the small amount of fat on the density can be ignored. Recalculating on this basis, the apparent specific volume found is 0.696. The agreement with the value given in Table 1, 0.696, is exact as it should be, since the complex : nitrogen factors used in the calculations for Table 1 were based on total solids results and involved the same corrections for serum protein and fat.

Reference has been made to a specific volume reported by Nichols *et al.* (12). These authors give total solids and densities for a skimmilk and for the casein-free serum obtained from it by ultrafiltration. Using their data, and calculating as above, the apparent specific volume obtained is 0.703. Nichols *et al.* corrected

TABLE 1

Apparent specific volume of the casein complex, calculated from data on progressively depleted samples of skimmilks, and the corresponding calcium: nitrogen and complex: nitrogen ratios; together with the serum nitrogen and the density values used in the calculations

Date	S.N.[a] gram per 100 g.	Serum density (24.8°/4°)	Density at 0.50% C.N. (24.8°/4°)	Ca/C.N.[a]	Complex/C.N.	A.S.V.[a]	Milk
October, 1946	0.165	1.02615	1.03605	0.182	6.83	0.692	Jersey
November, 1946	0.146	1.02532	1.03505	.....	(6.89)	0.699	Herd
December, 1946	0.140	1.02523	1.03528	.....	(6.89)	0.692	Jersey
September, 1954	0.167	1.02487	1.03450	.....	(6.89)	0.701	Jersey
August, 1955	0.119	1.02330	1.03300	0.182	6.77	0.696	Jersey[b]
August, 1955	0.140	1.02270	1.03240	0.184	6.90	0.703	Holstein[b]
Averages	0.146	1.02460	1.03438	0.183	6.86	0.6972[c]	

[a] S.N. = serum nitrogen; C.N. = casein nitrogen; A.S.V. = apparent specific volume.

[b] About 20% of the colloid was removed from these two skimmilks by preliminary centrifugation. The results, therefore, apply to intermediate- and small-particle sizes only.

[c] Standard deviation, 0.0039.

their serum total solids for the albumin-globulin held up on the filter, but they did not make a corresponding correction for the serum density. They give a specific volume of 0.665. Aside from the failure to correct the serum density, however, they also ignored hydration, and they obtained a value for total solids removed by simple subtraction of their corrected serum total solids from the skimmilk total solids.

Values Based on Density Measurements on Water Suspensions of Centrifugally Deposited Water-Washed Casein Colloids. The experiments previously reported (9, 10), have shown that the casein complex contained in the colloids centrifugally deposited from milk is not sensibly altered in composition by redispersing the deposit in distilled water and recentrifuging. The colloids are, however, thus washed free of lactose, and presumably washed free of serum salts. They are also, to a large extent, freed of ether-extractable substances which are always present in the unwashed deposits. They are not, however, freed of serum proteins.

Several twice-washed water suspensions of the casein colloids were prepared as indicated, analyzed for total nitrogen, casein nitrogen, calcium, and total solids, and the densities determined. In the calculations based on these experiments, the quantity h in Equation (1) was taken as referring to the total solids. The apparent specific volume of the total solids was then corrected for the contained serum protein by use of the relationship

$$V_{\text{complex}} = \frac{(T.S.)(V_{T.S.}) - (S.P.)(0.751)}{(T.S. - S.P.)}, \qquad (3)$$

where $T.S.$ means total solids, $S.P.$ means serum protein (serum nitrogen $\times$ 6.38), and $V_{T.S.}$ is the specific volume of the total solids as directly determined. The factor 0.751 is the specific volume given by Svedberg and Pedersen (16) for albumin and globulin.[4] In these calculations, since the solvent is water, hydration is disregarded, as is common practice. The results obtained in this way, with corresponding calcium : nitrogen and complex : nitrogen ratios, are given in Table 2. In the last three experiments, the complex : nitrogen ratios were not determined, but calculated from the calcium : nitrogen ratios (*cf.* Reference 10).

Calculations of the Specific Volume of the Casein Complex Based on the Specific Volumes of Its Components, for Minimum, Average, and Maximum Calcium : Nitrogen Ratios. The method of calculating specific volumes of proteins by adding the weights and the volumse of constituent groups and dividing the sum of the volumes by the sum of the weights was described by Cohn and Edsall (2). It has been used by McMeekin, Groves, and Hipp (11) for casein. The method is here employed for the casein complex.

The casein complex will be considered to be composed by adding the amounts of calcium caseinate, tricalcium phosphate, and trimagnesium phosphate equiva-

[4] A value 0.734 has recently been reported (3) for bovine serum albumin, which is commonly presumed to be identical with milk serum albumin. The best value for lactalbumin remains 0.751. In the absence of a new value for isolated serum albumin, 0.751 is used here. Substitution of 0.734 would affect the final result by less than 0.15%.

TABLE 2

Apparent specific volumes of the casein complex based on analyses and density determinations on suspensions of centrifugally deposited, water-washed colloids; and corresponding calcium: nitrogen and complex: nitrogen ratios[a]

Ca/C.N.[b]	Complex/C.N.	A.S.V.[b]	Milk	Particle-size fraction
0.198	6.87	0.696	Jersey	Composite
0.200	6.80	0.697	Jersey	Composite
0.209	6.85	0.698	Jersey,[c] 24 hr. at 4° C.	Composite
0.215	6.91	0.700	Jersey, 48 hr. at 4° C.	Composite
0.216	6.90	0.700	Jersey, 1 wk. at 4° C.	Composite
0.202	6.83	0.697	Jersey[d]	Small particles
0.204	6.96	0.692	Jersey	Intermediate
0.181	6.82	0.701	Holstein[e]	Small
0.196	6.86	0.698	Holstein	Intermediate
0.204	6.89	0.698	Holstein	Large
0.203 Average	6.87 Average	0.6977[f] Average		

[a] There is a discrepancy between the Ca/C.N. and Complex/C.N. values for the 1-wk. Jersey milk as between this table and the third part of Table 2 of the preceding paper on the nitrogen factor (10). The values given here are correct. The error in the previous paper has no effect on the final result.

[b] C.N. = casein nitrogen; A.S.V. = apparent specific volume.

[c] This and the following two samples were made from the same milk.

[d] This and the following sample were made from the same milk.

[e] This and the following two samples were made from the same milk.

[f] Standard deviation, 0.0024.

lent to 1 g. of nitrogen, using minimum, average, and maximum analyses previously reported (9). The three compositions, in grams and milliliters of the assumed components are given in Table 3.

The weight of calcium caseinate used in Table 3, 6.519 g., is equal to 6.45 + 0.0727 − 0.0037, the casein: nitrogen ratio, plus the grams of calcium equivalent to the phosphorus in casein, less the grams of hydrogen displaced by the calcium

TABLE 3

Minimum, average, and maximum calculated specific volumes of the casein complex in milk, based on analyses of liquid samples progressively depleted of the casein colloid by centrifuging

Components	Minimum Ca content found by analysis Ca/N = 0.181		Average Ca content found by analysis Ca/N = 0.206		Maximum Ca content found by analysis Ca/N = 0.232	
	(g.)	*(ml.)*	*(g.)*	*(ml.)*	*(g.)*	*(ml.)*
Calcium caseinate per gram nitrogen $\bar{V} = 0.715$	6.519	4.661	6.519	4.661	6.519	4.661
$Ca_3(PO_4)_2$ per gram nitrogen[a] $\bar{V} = 0.38$	0.280	0.106	0.344	0.131	0.410	0.156
$Mg_3(PO_4)_2$ per gram nitrogen[b] $\bar{V} = 0.38$	0.016	0.006	0.019	0.007	0.023	0.009
Totals	6.815	4.773	6.882	4.799	6.952	4.826
Specific volume *(ml/g)*	0.700_4		0.697_3		0.694_2	

[a] $Ca_3(PO_4)_2$ equivalent to the total calcium diminished by calcium equivalent to organic phosphorus.

[b] $Mg_3(PO_4)_2$ equivalent to 1/15th the total mols of calcium (*cf.* Reference 1).

(*cf.* Reference 10). The weight of tricalcium phosphate is that equivalent to the total calcium in the complex, less the 0.0727 g. of calcium assumed to be present as calcium caseinate. The weight of trimagnesium phosphate assigned is based on a mol ratio of magnesium to calcium of 1 to 15 (1).

The volumes, in Table 3, are the products of the weights of the components and the specific volumes. The specific volumes used are based on supplementary measurements and calculations.

The specific volume of calcium caseinate was determined by density measurements on filtered and centrifugally clarified solutions of freshly precipitated and washed casein in lime water. The average of ten values is 0.714, and the standard deviation, 0.004. Equation (1) was used. The specific volume was also estimated by starting with 6.45 g. of casein of specific volume 0.731, the value given by McMeekin, Groves, and Hipp (11), adding the equivalent weight and volume of calcium, and subtracting the weight and volume of hydrogen displaced. Calculating the specific volumes of calcium and hydrogen from the ionic radii 0.98 and 1.36 Angstroms, due to Zachariason (14), the specific volume thus obtained for calcium caseinate is 0.720. However, it is permissible to increase the ionic radius of the hydrogen by at least about 0.5 Angstroms, on the presumption that in the dissolved state the ester phosphate hydrogens of calcium caseinate are exposed and, when this is done, the specific volume found is 0.714. If the ionic radius of calcium is increased by a like amount the specific volume becomes 0.716. A similar calculation can be based on the specific volumes of ortho-phosphoric acid and calcium dihydration phosphate, 0.448 and 0.258, obtained from density data. Using these figures, the decrease in specific volume resulting when two hydrogens are replaced by calcium, in phosphoric acid, is found to be 0.706 cc. per gram of calcium. If the same decrease in volume occurs when two ester phosphate hydrogens of casein are replaced by calcium, then a simple calculation gives 0.715 for the specific volume of calcium caseinate.

The specific volume of tricalcium phosphate was determined by density measurements on suspensions prepared by mixing solutions of $Ca(OH)_2$ and $CaHPO_4$. The value 0.38 was obtained. By adding up the ionic volumes, the result is 0.36, and if the calcium is presumed to be in part exposed, a value of about 0.42 is obtained. The experimental value is used in Table 3. The specific volume of trimagnesium phosphate was not determined, but the value 0.38 was used. The amount of magnesium phosphate involved is so small that even a large error in the specific volume would have little effect on the final result.

The three specific volumes for the complex given in Table 3 are in good agreement with the experimental values. The purpose of these calculations is not, however, to show agreement between theory and experiment, but primarily to set limits of variability of the specific volume with composition. This variability is about 0.45% of the mean value.

In accordance with the method of calculation of the theoretical values, it is seen that the relationship between the apparent specific volume and the calcium : nitrogen ratio is not linear. In the useful range, however, between the

extreme calcium : nitrogen ratios, 0.181 and 0.232, found by experiment (9), it is approximately linear, and can be described by the equation

$$V_{complex} = 0.700 - (0.121)(Ca/C.N. - 0.181). \quad (4)$$

DISCUSSION

Assumptions Involved in the Calculations Based on Progressively Depleted Skimmilk Samples. These calculations are complicated by two factors: the centrifugal casein-free serum does not have the same composition as the serum or solvent phase of the skimmilk, and the disperse phase is not the anhydrous complex but a hydrate. Centrifugal casein-free serum has been partially depleted of serum protein and, possibly, small amounts of lactose and salts also, by centrifugation. It is for this reason that the true serum nitrogen concentration must be calculated from the analysis of the skimmilk, assuming a value for hydration; and an approximate density for this serum then taken from the density : total nitrogen plot, or calculated. Taking the December, 1946, experiment, Figure 1, for example, if the serum density were assumed to be that of the centrifugal casein-free serum, and the calculations carried out otherwise as indicated, assuming a hydration of 0.52 g. per gram, the apparent specific volume obtained would be 0.687 rather than 0.693, as given. If hydration were ignored also, i.e., assumed to be zero, then the apparent specific volume 0.701 would be obtained.

Hydration can be disregarded in determinations of apparent specific volumes for pure proteins dissolved in water, if the density of hydrate water is assumed to be the same as that of water in bulk. It is commonly disregarded also for solutions of proteins in dilute buffers. It can not be disregarded in the present case, because the density of the solvent differs a great deal from that of water. Again taking the December, 1946, experiment as an example, correcting serum densities as indicated but assuming hydrations of zero, 0.52 g. per gram, and 1.0 g. per gram, the apparent specific volumes found are 0.706, 0.693, and 0.679, respectively.

The assumption that the density : total nitrogen plots can be extrapolated to zero casein nitrogen may or may not be justified. In one of the experiments reported in Table 1, however, for August, 1955 (Jersey milk), 95% of the casein nitrogen was removed with no deviation in linearity of the density plot, and in other experiments in which densities were not determined, as much as 99% of the casein nitrogen has been centrifugally removed. Whether the extrapolation is justified or not, however, makes no difference in the calculations. The serum may be assumed to contain 5, 10, or 20% casein nitrogen, and when the calculations are carried out as indicated, the same apparent specific volume for the remaining complex is obtained. This could be predicted, since the specific volume : total nitrogen plots, like the density plots, are straight lines within the accuracy of the data.

Collected results. The 13 sets of apparent specific volume–calcium : casein nitrogen ratio values of Tables 1 and 2 were analyzed by the least-squares method, giving equal weight to all the data. The values obtained for the constants a and b

in the equation $V = a + b(Ca/N - 0.181)$ are 0.6974 and -0.007. Using only the data for washed colloids, Table 2, the constants 0.6986 and -0.058 are obtained. In the second case, the apparent specific volumes given by the equation at the minimum, average, and maximum calcium : nitrogen ratios, 0.181, 0.206, and 0.232 are 0.6986, 0.6971, and 0.6956, respectively. The data are too meager to establish a slope with any precision, but since the experimental average specific volume agrees so well with the theoretical value, 0.6973, it seems permissible to assume the slope to be the theoretical slope, -0.121, given in Equation (4). When this is done, and the sum of the squares of the deviations from a line having this slope is made a minimum, the value obtained for a, using all the data, is 0.6992; the apparent specific volumes at the minimum, average, and maximum calcium : casein nitrogen ratios are 0.6992, 0.6961, and 0.6930, respectively, and the standard error of estimate is 0.0033. If only the data for washed colloids are used, a becomes 0.6998, the three apparent specific volumes are 0.6998, 0.6968, and 0.6936, and the standard error of estimate is 0.0024.

These statistical results indicate that the constant a in Equation (4) should probably be about 0.700, as given; that the apparent specific volume for a complex of average composition (calcium : nitrogen, 0.206) is about 0.697; and that the extreme deviations to be expected in this value are about equal to the probable experimental error, 0.003.

Specific volume of the actual casein complex particle in milk. The apparent specific volume calculated here is the specific volume of the calcium caseinate–calcium phosphate complex. As it exists in milk or in any aqueous medium, this complex is hydrated and solvated. The specific volume of the actual dispersed particle or micelle is, therefore, determined by the contributions of the complex, the hydrate water, and the solvate liquid to the total weight and volume. These contributions can be calculated. For purposes of illustration, a complex of average composition will be assumed for which $V = 0.697$ in accordance with Equation (4). It will be further assumed that the hydration is 0.52 g. per gram; that the density of hydrate water is 0.9970 at 25° C.; that the ratio of the volume of the total particle to the weight of contained dry complex is about 3.1 to 1 (5); that the solvate liquid is identical with casein-free milk serum and has a density of 1.0264; and that no serum proteins or other solids are present, except those in the solvate liquid. Using these values, for the total particle:

$$V_{\text{particle}} = \frac{3.1}{1.000 + 0.52 + (3.1 - 0.697 - 0.522)\ (1.0264)} = 0.898 \qquad (5)$$

The corresponding density is 1.114. This is the density that would be used in calculations of particle sizes from sedimentation velocities by use of Stokes' law, for example. These hydrated, solvated colloids can be separated by centrifugation, and obtained as wet jelly-like deposits (*cf.* Reference 9), with some inclusion of serum proteins in excess of those in the solvate liquid, and with variable entrainment of excess milk serum, depending on the centrifugal force used. Densities of such wet deposits determined by displacement of xylene approach

the value 1.114 as the centrifugal force is increased and the amount of entrained serum approaches zero. Such experiments will be reported in detail later.

CONCLUSIONS

1. Skimmilks were progressively depleted of casein colloids by centrifuging, supernatant liquids and deposited fractions being thus obtained. The deposits were twice washed by redispersing in water and recentrifuging. Densities were determined on the supernatant liquids and on water suspensions of the washed colloids.

2. Apparent specific volumes were calculated by the equation of Svedberg and Chirnoaga. In the calculations based on densities of supernatant liquids the mathematical treatment is novel in that the apparent specific volume of the casein complex hydrate considered as the solute is first calculated, and the apparent specific volume of the unhydrated complex is then calculated from this value. This procedure is necessary because the solvent is not water in this case but milk serum of density appreciably different from that of water. In the calculations based on densities of water suspensions of washed colloids this refinement is not necessary.

3. For six sets of data on supernatant liquids the average apparent specific volume found for all fractions for the casein complex was 0.697 ± 0.0038. For ten sets based on water suspensions of washed colloids the average value found was 0.698 ± 0.0018. The corresponding average calcium : nitrogen ratios were 0.183 and 0.203.

4. The apparent specific volume of the complex calculated from the specific volumes of its components, as indicated by analyses previously reported, is a function of the calcium : nitrogen ratio in the complex, and is given by the equation

$$\text{A.S.V.} = 0.700 - 0.121\ [(\text{Ca/N}) - 0.181]$$

This equation is in statistical agreement with the experimental results. For a large number of samples the minimum, average, and maximum calcium : nitrogen ratios found are 0.181, 0.206, and 0.232. These values used in the above equation give theoretical, and actual, apparent specific volumes of 0.700, 0.697, and 0.694, respectively, with a probable error of $\pm$ 0.003 or about 0.4%. These values apply to the total complex and to the complex in any particular size fraction, for Jersey or Holstein milk.

5. It is emphasized that the apparent specific volumes calculated are for the casein complex and not for the total colloidal particle as it exists in milk. The specific volume of the actual dispersed particle can be calculated from the specific volume of the complex by taking hydration and solvation into account. An example of such a calculation is given.

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INHIBITION OF LIPASE AND LIPOLYSIS IN MILK[1]

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SUMMARY

The storage stability of milk lipase was markedly increased by the addition of glutathione, hydroquinone, and potassium thiocyanate. These results indicate that the loss of lipase activity on aging milk results mainly from oxidative changes. The greater effectiveness of glutathione in stabilizing milk lipase may indicate the importance of sulfhydryl groups for this enzyme activity. The heat inactivation of milk lipase followed first-order kinetics. The pH of optimum stability was in the range of 6.6–7.6. Milk lipase was very sensitive to photo-inactivation and to oxidizing agents. The stability of milk lipase to heat, light, hydrogen peroxide, and copper, like storage stability, was greater in whole milk than in skimmilk. It is suggested that the sulfhydryl groups in the membrane material of the fat globules are preferentially oxidized, thus protecting the lipase system.

The inhibition of lipolysis was greatly decreased when inhibitors were applied after activation treatments of lipolysis. The evidence indicates that this protection of the lipase system may be due to a specific enzyme-substrate combination which results from the activation treatments. It is concluded that the lipase system is affected by the activation treatments of lipolysis.

Basic information on the stability of milk lipase is of practical and fundamental importance. Thus far, heat treatments have been used exclusively to inactivate milk lipase. Other inactivation treatments might be useful in dairy processes where milder heat treatments are desirable. Work on the isolation and purification of lipase from milk requires a knowledge of conditions for maximum stability. The inhibition of different activation treatments used to induce lipolysis in milk also may shed further light on the mechanism involved in the activation of lipolysis in milk.

Studies on the inactivation of milk lipase were reviewed by Herrington (7). The results of those studies were complicated by uncertainties involved in the methods used to determine this enzyme system. A previous discussion of this problem led to standardization of a procedure to measure the lipolytic activity of milk toward milk fat (5). Failure to consider the effect of the instability of the lipase system on the aging of milk prior to activation (17) may have resulted in additional complications. In a previous study (6), using milk fat and simpler esters as substrates, we have shown that lipase is more stable to aging, heat, acid, alkali, light, oxidizing agents, and formaldehyde than the simple esterases of the same milk. It was also observed that lipase was more stable to aging in whole milk than in skimmilk. Therefore, the presence or absence of milk fat may be an important variable in studies on milk lipase stability.

The mechanism by which different activation treatments induce lipolysis in normal milk is not well understood. There is controversy as to whether the

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activation treatments induce lipolysis by affecting the lipase system as well as the substrate. Dorner and Widmer (1) have shown that lipolysis resulted when raw milk was added to homogenized milk previously heated to 75° C. to destroy lipase. This indicates that homogenization affected the substrate only to induce lipolysis. Larsen *et al.* (12) found that maximum lipolysis resulted in a one-to-one mixture of unhomogenized raw milk and homogenized pasteurized milk. They concluded that the development of lipolysis depended equally on the concentration of lipase added and the increased surface or "surface activation" afforded by homogenization. Dunkley and Smith (2) found no correlation between tributyrinase and temperature-induced lipolysis. They considered that activation treatments (temperature-fluctuation, agitation, and homogenization) affect the substrate to induce lipolysis. This view recently has been shared and emphasized by Herrington (8). However, a study of activation of lipolysis by shaking or foaming (17) has indicated that this treatment affects the lipase system.

The present investigation deals with the stability of milk plasma lipase toward different inhibiting agents in the presence and absence of globular milk fat. The effect of different inhibiting agents on lipolysis was studied when applied before and after activation, in order to gain an insight on the relative changes in the enzyme-substrate relationship brought about by activating treatments.

EXPERIMENTAL PROCEDURE

Freshly drawn milk, mixed or from individual cows, was obtained in amber glassware and processed immediately after milking. The lipase concentration was determined in the skimmilk obtained by centrifugation at 37° C. The method used for assaying lipase activity toward milk fat has been described previously (5). Lipolysis was activated in milk by the following treatments:

Homogenization. One volume (25 ml.) of raw milk was mixed with one volume of pasteurized homogenized milk (of the same fat content) in 125-ml. Erlenmeyer flasks. The same supply of pasteurized homogenized milk was used for all experiments.

Shaking: A 50-ml. portion of milk in a 125-ml. Erlenmeyer flask was brought to 37° C. in a Warburg apparatus and shaken for 30 min. at 150 double oscillations per minute.

Temperature activation: The milk in 50-ml. portions was aged at 0° C. for 4 hr., then placed in a 31.0° C. bath and mixed gently at regular intervals. When the temperature of the milk reached 30° C., it was allowed to stand at this temperature for 15 min., with occasional mixing.

After activation, the milk was cooled in ice water to 5° C. and stored 24 hr. at 0–5° C. The extent of lipolysis was measured by determining the development of free fat acidity (F.F.A.) (4).

RESULTS

Inhibition of Milk Lipase.

Aging. The variation in the stability of milk lipase on aging was studied in five different mixed milks and the milk from ten cows. After aging at 0–5° C. for 24 hr. after milking, the inactivation of lipase varied from 15 to 39% in the

mixed milks and from 6 to 50% in the milk from individual cows. In view of this variation, all subsequent experiments were performed with milk obtained within 1 hr. of milking.

Several experiments were carried out to obtain an insight on the mechanism for loss in lipase activity on aging milk in the cold. The sulfhydryl groups of enzyme proteins generally are considered most susceptible to oxidation. Therefore, attempts were made to determine whether any protection could be afforded by aging milk in the presence of glutathione. Fresh milk was aged in the presence and absence of glutathione and less specific reducing agents, including hydroquinone and potassium thiocyanate. All reducing agents were added at the rate of 5 ml. of 0.01 *M* solution per 50 ml. of milk. Appropriate controls were run during activity determinations, to take into account any possible activation from the presence of the reducing agents in the milk. None was detected by these agents during the short reaction periods used in the assays.

The results (Table 1) show that the storage stability of milk lipase is appreciably increased by reducing agents. A marked protective effect of glutathione is evident in milk stored for 24 and 48 hr. The other reducing agents do not show significant effect until after 48 hr. of storage. It is concluded that a considerable portion of the loss in lipase activity of milk and aging in the cold can be traced to oxidative inactivation of the enzymes. Part of this inactivation may be attributed to oxidation of sulfhydryl groups.

Since milk lipase previously had been found to be more stable to aging in whole milk than in skimmilk (6), the relative effectiveness of the reducing agents used was determined in the presence or absence of milk fat globules (4% milk fat). The increase in stability afforded by these agents was not affected by the milk fat. An experiment was carried out to determine whether pasteurized cream added to skimmilk at different levels would affect the stability of milk lipase. Although, again, the storage stability of lipase was greater in whole milk than in skimmilk, it was not affected in the latter by increasing the milk fat concentration. Therefore, the original environment in whole milk with respect to fat content appears to be optimum for the stability of milk lipase.

Heat. Heat inactivation of milk lipase first was studied by subjecting milk to different temperatures for a certain time. The procedure used to heat the

TABLE 1
Effect of reducing agents on the storage stability of milk lipase

	Per cent inactivation Length of storage at 0–5° C:		
Reducing agents	24 hr.	48 hr.	96 hr.
Experiment I			
Control milk	39	56	70
Plus glutathione	1	1	17
Experiment II			
Control milk	23	54	67
Plus glutathione	6	20	44
Plus hydroquinone	25	47	41
Plus K thiocyanate	21	34	43

milk was the same as that described previously (6). The results (Table 2) show that almost complete inactivation is attained by heating the milk 15 min. at 60° C.

The kinetics of heat inactivation of milk lipase was followed by heating milk at 50° C. for varying periods. The results (Figure 1) show a linear relation between the log of activity toward milk fat and time of heating. Therefore, the heat inactivation of milk lipase, like that of most enzymes (15), follows a first-order reaction. The first-order specific reaction rate-constant was calculated to be 0.0423 minute^{-1} for the hydrolysis of milk fat in the above milk.

To determine the possible effect of milk fat globules on the heat stability of milk lipase, whole milk and skimmilk derived from it were held 15 min. at 50° C. The whole milk was separated before assaying. The inactivation was 36% in the whole milk and 51% in the skimmilk. Therefore, to a certain degree, milk lipase is protected against heat inactivation by the globular fat in the whole milk.

Acid and alkali. The effect of pH on the stability of milk lipase was studied

TABLE 2

Effect of heating skimmilk at different temperatures for 15 min. on the lipase activity

Heating temperature	Activity	Inactivation
(° C.)	*(units per 100 ml.)*	*(%)*
Control	3.78	....
40	2.71	28
45	2.14	43
50	2.09	45
55	0.47	88
60	0.13	97

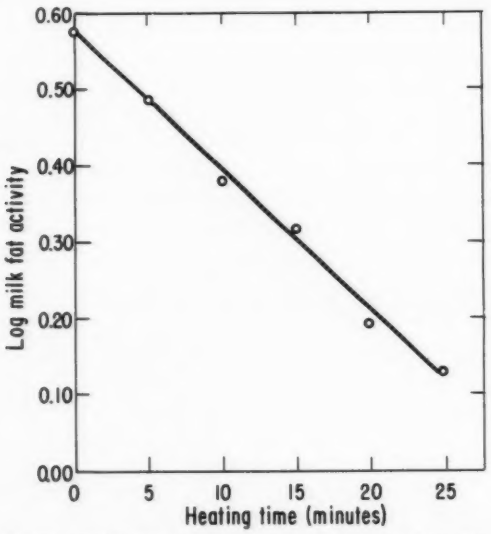

FIG. 1. Rate of heat inactivation of milk lipase at 50° C. Milk fat activity: units per 100 ml. milk.

by determining the pH-stability curve for the hydrolysis of milk fat. Milk was allowed to stand at different pH's for 1 hr. at 37° C. before assaying for activity. No buffer was used, and the final pH observed after treatment was taken as the experimental pH of the milk. The reaction mixtures contained sufficient alkali or acid to bring pH to the optimum for the reaction (pH 8.9), as determined on separate aliquots of corresponding milk samples.

The results obtained with two different milks are given in Figure 2. The pH of maximum stability was in the range of 6.6–7.6. Therefore, normal milk is in an optimum pH range for the stability of milk lipase. Inactivation was about 50% when the pH was lowered to 6. Acid and alkali inactivation of milk lipase was not affected by the presence of milk fat globules; loss in activity was the same in whole milk and skimmilk subjected to these treatments.

Trypsin. Among various commercial preparations of proteolytic enzymes, trypsin proved the most effective in inactivating milk lipase at the normal pH of milk; pepsin had no effect. The effect was studied on treating skimmilk with different concentrations of crystalline trypsin.[3] Portions of 100 ml. of skimmilk were incubated 1 hr. with 5, 7, 10, and 20 p.p.m. of trypsin in a water bath at 37° C. with gentle agitation. These concentrations of trypsin produced, respectively, 47, 50, 83, and 100% inactivation of milk lipase. In another experiment, fresh skimmilk was incubated with 7 p.pm. of trypsin at 37° C. for different periods. Inactivation reached 32% after 30 min., and 50% after 60, 90, or 120 min. of incubation. Trypsin inactivation of milk lipase was not affected by the presence of globular milk fat in the milk.

Light and oxidizing agents. The photo-inactivation of milk lipase was studied

[3] Obtained from the Armour Laboratories, Chicago, Ill. (1 mg. equivalent to 2,500 "Armour units").

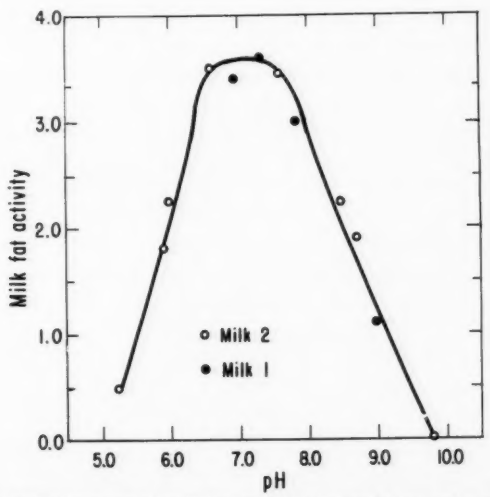

FIG. 2. The pH-stability curve of milk lipase. Milk fat activity: units per 100 ml. milk.

by exposing layers of skimmilk to bright sunlight and diffuse daylight (northern exposure) for varying periods. When a layer of skimmilk 1 cm. thick, in a Petri dish 14 cm. in diameter, was exposed to direct sunlight at room temperature, an 84% loss in the activity of milk toward milk fat occurred within 5 min. After 10 min., the inactivation reached 96%. The inactivation of a like layer of milk by exposure to diffuse daylight is shown in Table 3. Temperature was not critical, since exposure at 0° C. under the same conditions resulted in essentially the same inactivation. The course of photo-inactivation appears to follow an autocatalytic reaction.

TABLE 3

Effect of exposing skimmilk to diffuse daylight on the lipase activity

Exposure time	Activity	Inactivation
(min.)	*(units per 100 ml.)*	*(%)*
0	3.97	....
15	3.84	3
30	2.75	31
45	1.73	56
60	1.15	71

Milk lipase was markedly protected against photo-inactivation by the presence of milk fat. When fresh, whole milk and the same milk, separated, were exposed to diffuse daylight 1 hr., lipase activity was reduced 48 and 70%, respectively.

The oxidizing agents studied were copper and hydrogen peroxide. Skimmilk was allowed to stand with different concentrations of copper sulfate and hydrogen peroxide solutions and then assayed for lipase. Treatments with 5–20 p.p.m. of Cu^{++} for 15 min. at room temperature, with occasional mixing, resulted in 7 to 17% inactivation. Incubation with 5 p.p.m. of Cu^{++} for 1 hr. at 37° C. produced 69% loss of milk lipase activity. The results obtained with different concentrations of hydrogen peroxide are presented in Table 4. Inactivation was almost complete with 10 p.p.m. hydrogen peroxide.

The effect of milk fat on the relative inactivation by copper and hydrogen peroxide was tested by allowing these agents to act on whole milk and skimmilk. Each milk was treated with 2 p.p.m. H_2O_2 for 15 min. at room temperature, and with 5 p.p.m. Cu^{++} for 30 min. at 37° C. The whole milk was separated just

TABLE 4

Effect of hydrogen peroxide on the lipase activity of skimmilk

Concentration[a]	Activity	Inactivation
(p.p.m.)	*(units per 100 ml.)*	*(%)*
0	3.92	....
1.0	2.71	31
2.5	1.61	59
5.0	0.67	83
7.5	0.42	89
10.0	0.39	92

[a] Treatment: Skimmilk allowed to stand at room temperature with hydrogen peroxide for 15 min.

before assaying. Losses in lipase activity in the hydrogen peroxide–treated whole milk and skimmilk were 34 and 55%, respectively. With the copper-treated whole milk and skimmilk, the inactivation reached 19 and 36%, respectively. Therefore, under the experimental conditions, globular milk fat exerts a marked protective effect on the lipase against these oxidizing agents. This protective effect of milk fat corresponds to that observed in the inactivation of milk lipase by aging, heat, or light.

Inhibition of Lipolysis in Milk.

The experiments were performed as follows: Fresh mixed milk was cooled to 0–5° C. and allowed to age at this temperature for 4 hr. after milking. It was then divided into four fractions. Fraction I was kept cold and constituted the nonactivated control. Fraction II was the first fraction subjected to inactivation treatment. Lipolysis was induced in Fractions II, III, and IV—by homogenization, shaking, and temperature activation. Immediately after activation, Fraction III was subjected to the same inhibition treatment as Fraction II. Fraction IV constituted the noninhibited activated control. After activation, all samples were cooled to 5° C. and stored at 0–5° C. Free fat acidity was determined in all samples after aging 24 hr. at this temperature. The extent of lipolysis in all fractions was taken as the increase in free fat acidity over that in Fraction I.

The inhibition treatments were applied as follows: Heat inactivation was carried out by heating the milk to 50° C., holding it there for 15 min., and immediately cooling to 10° C. Photo-inactivation was applied by subjecting a layer of 1 cm. of milk to diffuse daylight for 1 hr. at room temperature. Inactivation by trypsin and copper was done by incubating the milk 1 hr. at 37° C. with 7 p.p.m. crystalline trypsin and 2 p.p.m. copper, respectively. Hydrogen peroxide and formaldehyde were applied, at 2 p.p.m. for 50 min. and 0.04% for 1 hr., respectively, at room temperature.

Representative results from several experiments are given in Table 5. The relative inhibition of lipolysis varied widely with each activation treatment. When inhibition was applied before activation, the lipolysis induced by temperature activation was the most affected. On the other hand, lipolysis from homogenization and shaking was affected similarly by heat, light, hydrogen peroxide, and copper. These differences in inhibition of lipolysis by homogenization and shaking on one hand, and temperature activation on the other, agree with those obtained with inactivation by aging milk in the cold (17), in suggesting that different mechanisms may be involved in the activation of lipolysis in each case.

The most interesting aspect of these results is the reduced effectiveness of inhibition treatments applied after activation. This is particularly evident in the case of activation by shaking, where no decrease in lipolysis occurred from inhibition treatments applied after activation (the decrease with trypsin was negligible). It appears that, as a result of the activation treatments of lipolysis, the lipase system becomes significantly protected against inhibition. This protection may be related to an enzyme-substrate association resulting from the acti-

TABLE 5

Inactivation of lipolysis in milk by different agents

Inhibition treatments	Activation treatments	Lipolysis			Decrease in lipolysis	
		Non-inhibited activated controls	Inhibited before activation	Inhibited after activation	Inhibited before activation	Inhibited after activation
		(free-fat acidity)			(%)	
Heating	Homogenization	7.40	4.36	3.61	41	51
	Shaking	10.3	7.25	10.8	30	0
	Temperature	3.91	0.37	0.57	91	85
Light	Homogenization	7.40	4.22	6.64	43	10
	Shaking	10.3	6.03	12.7	42	0
	Temperature	3.91	1.03	1.79	74	54
Trypsin	Homogenization	10.6	8.60	9.60	19	9
	Shaking	14.5	0.99	13.6	93	6
	Temperature	1.90	0.08	2.35	96	0
Hydrogen peroxide	Homogenization	8.00	5.54	6.14	31	23
	Shaking	8.90	6.14	9.80	31	0
	Temperature	2.20	0.91	1.14	59	48
Copper	Homogenization	8.00	4.32	7.44	46	7
	Shaking	8.90	5.93	9.80	33	0
	Temperature	2.20	0.38	1.60	83	27
Formaldehyde	Homogenization	8.00	4.48	6.90	44	14
	Shaking	8.90	0.84	10.0	91	0
	Temperature	2.20	0.0	0.76	100	66

vation treatments. In the case of shaking, this relationship is such that the enzyme system is completely inaccessible to any of the inhibiting agents used.

The relative inhibition by heat, light, hydrogen peroxide, and copper in the activated samples is evidently related to the extent of lipolysis in the activated controls (not subjected to inhibition). The more lipolysis takes place in the activated milks, the less inhibition takes place when these agents are applied after the activation treatments. All these inactivating agents would denature the lipase proteins. It is apparent, therefore, that the lipase action is directly related to the accessibility of the enzyme system to these denaturing agents. The accessibility of the enzyme system could be related, on this basis, to the extent of its association with the milk fat substrate necessary to initiate and promote lipolysis.

An attempt was made to determine whether the concentration of lipase in the skimmilk from activated milks might be affected in any way by changes in the enzyme-substrate relationship resulting from different activation treatments. Fresh whole milk was subjected to the three activation treatments; the activated milk samples and the nonactivated control were aged at 0–5° C. for 24 hr. The extent of lipolysis was measured in an aliquot portion of each milk, the remainder being separated and the lipase concentration determined in the skimmilk. Since the efficiency of separation might vary in the activated milk, a Servall supercentrifuge[4] was used at 4,000 g. for 10 min., to obtain the skimmilk.

The results of this experiment are presented in Table 6. The zero-time read-

TABLE 6
Lipase concentration of milk after activation of lipolysis by different treatments

Enzyme sources	Zero-time reading	Activity in skimmilk	Lipolysis in milk
	(ml. 0.020 N KOH)	*(units per 100 ml.)*	*(F.F.A.)*
Control milk, not activated	0.20	3.51	0
1 volume raw milk plus 1 volume pasteurized homogenized milk	0.32	(2.64)[a]	8.47
Shaken milk	0.37	2.12	12.2
Temperature-activated milk	0.30	2.02	3.41

[a] Calculated value: $1.32 \times 2 = 2.64$, to take care of dilution of raw milk with pasteurized milk.

ings are reported, to compare the relative amounts of free fatty acids present initially in the skimmilk resulting from lipolysis. A marked decrease in lipase concentration is evident in the skimmilk from activated milks as compared to that from the nonactivated milk. The results indicate a combination or adsorption of lipase with the activated substrates, which would decrease the enzyme concentration in the skimmilks. The relatively small free-fat acidity in the skimmilks would not account for this decrease. The decrease in activity in the skimmilk does not correspond to the extent of lipolysis in the respective activated milks.

DISCUSSION

The relative instability of milk lipase was confirmed in this study. This was shown by the appreciable loss of activity resulting from aging skimmilk in the cold. The variation found in this loss of activity in several milks corresponds to that observed by Peterson *et al.* (13) for milk tributyrinase activity. The loss of stability of milk lipase was shown to result mainly from oxidative changes, since reducing agents greatly prolonged the storage stability of this enzyme system. These results are in agreement with early findings of Krukovsky and Sharp (11), that in the absence of dissolved oxygen, milk lipase inhibition by dissolved copper is eliminated and the thermal inhibition of this enzyme considerably diminished. Similarly, the removal of oxygen diminished the photoinhibition of milk tributyrinase (9) and increased the stability of an esterase in milk (3). The relatively great effectiveness of glutathione in reducing the loss of activity on aging, observed in the present study, provides a clue that sulfhydryl groups may play an important part in the activity of milk lipase. The requirement of sulfhydryl groups for the activity of a pancreatic lipase preparation was shown by Singer and Barron (14).

One interesting feature of the results on the stability of milk lipase was the marked protection afforded by the fat globules when the milk was subjected to aging, heat, light, and oxidizing agents. An explanation of the greater stability of milk lipase toward these inhibiting agents in milk than in skimmilk may lie in the relatively high concentration of sulfhydryl groups in the membrane material surrounding the fat globules (10). One may assume that these groups

[4] Model SS-1, supplied by Servall, Inc., New York.

may be preferentially oxidized, thus affording some protection to the oxidizable constituents of milk plasma lipase.

The most notable aspect of the results of inhibition of lipolysis was the markedly greater resistance of the lipase system to inhibiting agents in activated milk samples than in nonactivated ones. There is definite indication that the milk lipase system is affected by the activation treatments used to induce lipolysis. The lipase system appears to become intimately associated, by adsorption or redistribution, with the milk fat substrate, as evidenced by the decreased lipolytic activity in the skimmilk from activated milks as compared to that from the nonactivated control milk (Table 6). The results of this study conform to the mechanism postulated previously for the activation of lipolysis by shaking or foaming (17), which is based on lipase as well as substrate activation. Similarly, the lack of formaldehyde inhibition observed for spontaneous lipolysis (16, 19) could be attributed to the specific adsorption of naturally active lipase on the membrane material (membrane lipase) (18), making this enzyme system unavailable to the inhibitor.

The results emphasize the importance of two factors in studies on the stability and inhibition of milk lipase: 1. The presence or absence of globular milk fat in the system, and 2. The degree of activation of the milk fat substrate. Failure to consider these two variables may have contributed to the variation in inhibition of milk lipase by different agents reported in the literature (7).

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RELATION OF NATURAL COPPER IN MILK TO INCIDENCE OF SPONTANEOUS OXIDIZED FLAVOR[1,2]

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SUMMARY

Milk as it is produced by the cow contains a low but variable concentration of copper (natural copper). Results are presented that show a relatively high concentration (*ca.* 0.2 p.p.m.) of natural copper in early lactation milk, and a level of about 0.02–0.04 p.p.m. for most cows during the remainder of a lactation period. These results are similar to others reported recently.

No difference was observed between pasture and dry feed in the natural copper concentration in the resulting milk. When cows on dry feed were drenched with large (10-g.) doses of copper sulfate, there was an increase in the natural copper content of their milk and in the incidence and intensity of spontaneous oxidized flavor.

For cows on an alfalfa hay–grain concentrate ration, and not in early lactation, a highly significant correlation was observed between the concentration of natural copper in milk and the incidence and intensity of spontaneous oxidized flavor.

It is concluded that the copper present in milk as it comes from the cow is an important catalyst of oxidized flavor.

Copper is an effective catalyst of oxidized flavor in milk, but it has not been established whether the small amount normally in milk (natural copper) is important to development of the defect. The principal purpose of this study was to investigate the relation of natural copper concentration to the incidence of spontaneous oxidized flavor in milk produced by cows on alfalfa hay–grain concentrate ration.

Values reported for the natural copper content of milk are variable, with early workers obtaining higher results. The differences appear to be attributable to improvements in analytical technics (1) and to reduced contamination of samples. Koppejan and Mulder (6) and Menger and Mulder (8) indicated the variations that may be expected in milk samples taken from individual cows at different stages of lactation. Natural copper in milk is high (as high as 0.2 p.p.m.) during the first days of a lactation period, and gradually declines to normal levels (0.02–0.04 p.p.m.) during the first 2 mo.

The natural copper content of milk generally is not considered to be influenced by the cow's feed or copper intake (4, 8). However, Davis (2) stated that the copper content of milk can be almost doubled by a large increase in a cow's copper intake.

EXPERIMENTAL PROCEDURE

Experimental cows. The cows used in this study were a group of 20 Holsteins maintained continuously (except for a week on pasture) on a dry ration con-

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sisting of alfalfa hay and a grain concentrate mixture. They were given special attention, to prevent and control mastitis, with infrequent medication. Samples were taken during one to four lactations of the individual cows.

Sampling procedure. The cows were milked by machine, using stainless steel bucket units. A representative portion was removed from the weighing bucket with a stainless steel dipper and placed in a glass sample bottle. Within about 1 hr. after milking, the samples were divided into separate portions for flavor and copper examination and placed in a refrigerator at about 3° C.

Determining susceptibility to oxidized flavor. Two 100-ml. portions of milk were measured into glass milk bottles, and 0.1 p.p.m. of copper (as copper sulfate solution containing 10 mg. of copper per liter) was added to one portion. Both portions, stored at about 3° C., were examined for oxidized flavor by two scorers after two and five days. Samples were presented to the scorers with coded numbers and in random order. Intensity of oxidized flavor was evaluated as follows: 0—none; 1—questionable to very slight; 2—slight but consistently detectable; 3—distinct or strong, and 4—very strong. The results are averages of scores by the two scorers.

For convenience, the milks are arbitrarily divided into three classes (12): Spontaneous—milks without added copper that received a score of one or more after storage for five days; susceptible—milks with 0.1 p.p.m. of added copper that received a score of one or more in five days; and resistant—milks containing 0.1 p.p.m. of added copper that did not develop oxidized flavor (score less than one) in five days.

Determination of copper. The following procedure for determining copper was adapted from several previously described (3, 5, 7, 9).

Duplicate samples of milk were weighed into 150-ml. Pyrex beakers, and to facilitate drying about 4 ml. of 2.4 *N* hydrochloric acid was added with stirring. The samples were dried on a shielded water bath at 80–90° C. and ashed in a muffle furnace controlled to maintain a temperature of 500–525° C. The temperature was raised slowly, and when the samples were nearly white (usually about 12 hr.), they were cooled, and 2 ml. of concentrated nitric acid was added by running it down the sides of the beakers. The acid was evaporated by heating gently over a flame, the beakers were returned to the furnace for about 1 hr. at 500–525° C., removed, and allowed to cool.

Ten milliliters of 2.4 *N* hydrochloric acid was added with rotation to wet the entire interior surfaces of the beakers, the solutions were heated over a flame to boiling, and transferred to extraction flasks (75- to 100-ml. capacity, with glass stoppers, suitable for centrifuging). This procedure was repeated twice more, using 5-ml. portions of acid. Fifteen milliliters of ammonium citrate solution was added, followed by one drop of 1% ethanolic phenolphthalein solution and concentrated ammonium hydroxide to a faint pink end-point (pH about 9). This step prevented precipitation of phosphate and interference from ferric iron Two milliliters of 2% aqueous sodium diethyldithiocarbamate solution was added, the flasks were shaken vigorously, and 15 min. was allowed for completion of reaction.

Ten milliliters of isoamyl acetate was added and the flasks were agitated for extraction by inverting (in a rack) at a rate of 40 times per minute for 5 min. The flasks were then centrifuged for 5 min. at 600 r.p.m. About 4 ml. of the isoamyl acetate layer was transferred to a cuvette, and the optical density at 435 mμ was determined with a Beckman Model DU spectrophotometer, slit width 0.06 mm. A blank was prepared with each group of samples using 20 ml. of 2.4 *N* hydrochloric acid instead of the ash solution. The copper concentration was estimated from a standard curve prepared to cover a range of 0–1.5 μg. per milliliter of isomayl acetate.

All glassware used for the samples and in the analysis was immersed at least 5 min. in hot (60–80° C.) 1:4 nitric acid and rinsed with double-distilled water.

Double-distilled water was used for preparing reagents. The ammonium citrate solution was prepared by dissolving 430 g. dibasic ammonium citrate in about 300 ml. of water, adding 200 ml. of concentrated ammonium hydroxide, making the total weight up to 1,000 g. with water, and removing contaminating copper by adding 10 mg. of diethyldithiocarbamate and extracting 1–3 times with 100-ml. portions of isoamyl acetate. The sodium diethyldithiocarbamate solution was extracted with isoamyl acetate or carbon tetrachloride to remove contaminating copper and was stored in the dark. The isoamyl acetate used for extraction was the fraction with a boiling range of 136–142° C., that was distilled from either technical-grade product or solvent that had been used in previous copper determinations.

Other precautions necessary for reliable results have been discussed elsewhere (10, 11). Recovery experiments in which 5 μg. of copper was added to 100 g. of milk gave 90–110% recovery. The standard deviation of the differences between 261 duplicate determinations was 0.007 p.p.m.

RESULTS AND DISCUSSION

Stage of lactation. The copper determinations of milk samples taken throughout the lactation periods of three cows are presented (Figure 1). Day-to-day variations during the early part of another lactation period are also illustrated (Figure 2). The levels and trends illustrated by these data are representative of those for the other cows in this study, and are similar to results reported elsewhere (6, 8). Copper concentration was high in early lactation, but decreased (at a rate varying from cow to cow) to a more constant level during mid- and late lactation. The milk from most cows reached a level that was normal and characteristic for the cow during the first 2 mo. of lactation. For an individual cow, the normal level was in most cases between 0.02 and 0.04 p.p.m. and appeared to be a characteristic that was relatively constant in successive lactations.

Milks produced early in lactation were variable with respect to oxidized flavor: some developed oxidized flavor spontaneously, whereas others with high copper contents were very resistant.

Feed. The cows used in this study had been maintained on a dry ration consisting of alfalfa hay and a grain-concentrate mixture for about 2 yr., then were fed on pasture for a week. Flavor scores and copper determinations for milk samples taken before, during, and after this week are presented (Table 1), to

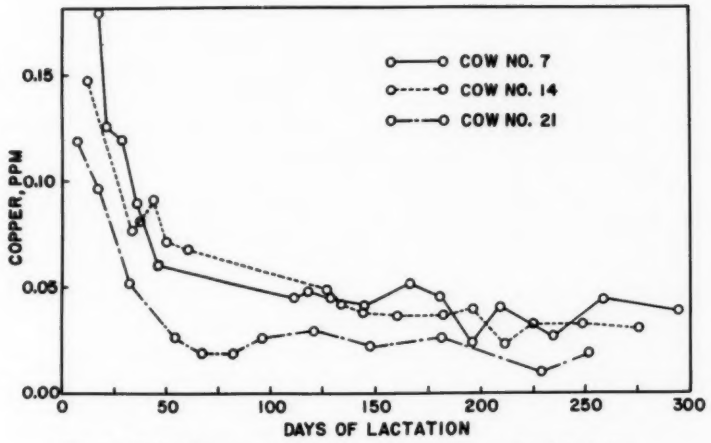

FIG. 1. Natural copper in milk of three cows throughout lactation.

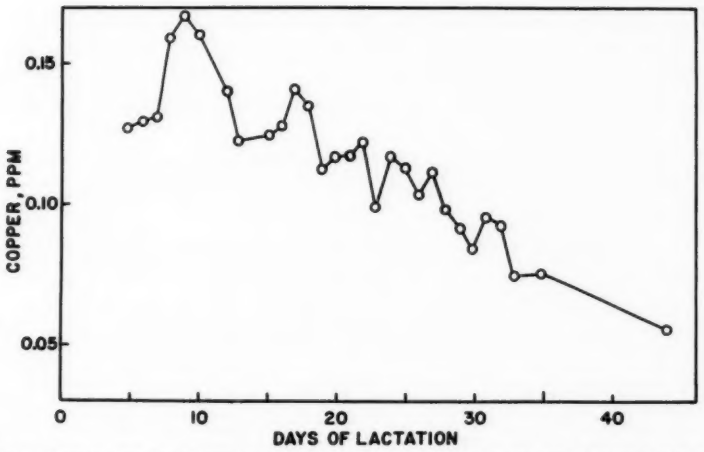

FIG. 2. Day-to-day variations in natural copper in milk from one cow during early lactation.

illustrate the effects of this change of feed. The data are arranged in decreasing order of the number of days each cow had been in lactation before pasture feeding. To facilitate consideration of the influence of stage of lactation on the natural copper content of milk, the data were averaged as two groups, divided arbitrarily on the basis of whether the cows had been in lactation for 60 days.

The data for copper-induced oxidized flavor are, in general, consistent with the common observation that pasture feeding reduces the susceptibility of milk to oxidized flavor, but individual cows varied in their response (note particularly No. 19). The incidence and intensity of copper-catalyzed oxidized flavor showed a marked decrease by the time the cows had been on pasture for five days, and

TABLE 1

Influence of pasture feeding on susceptibility of milk to oxidized flavor and natural copper content of milk

Cow No.	Days in lactation[a]	Time in relation to pasture feeding: 20 days before — Flavor score[b] — No Cu added	0.1 p.p.m. Cu added	Natural copper	1 day on — Flavor score — No Cu added	0.1 p.p.m. Cu added	Natural copper	5 days on — Flavor score — No Cu added	0.1 p.p.m. Cu added	Natural copper
				(p.p.m.)			*(p.p.m.)*			*(p.p.m.)*
10	315	1.0	3.0	0.028	0	4.0	0.035	0	0	0.035
11	268	1.0	2.0	0.050	0	2.8	0.041	0	0	0.040
16	243	0	3.8	0.042	1.5	3.8	0.043	1.0	0.8	0.039
21	240	0	3.3	0.025	0	3.3	0.022	0.5	1.8	0.038
9	174	0	3.5	0.030	0	3.8	0.043	0	1.0	0.092
15	158	0	3.5	0.013	0	3.8	0.015	0	2.0	0.014
12	124	0	2.5	0.039	0	2.0	0.040	0	0	0.028
13	123	0	3.3	0.030	0	2.8	0.034	0	1.5	0.017
19	115	2.5	4.0	0.040	3.2	4.0	0.066	0	3.3	0.035
20	77	0	2.8	0.063	1.5	2.8	0.048	0.5	0	0.034
8	73	0	1.5	0.038	0	2.0	0.029	0	0	0.023
Average		0.4	3.0	0.036	0.6	3.2	0.038	0.2	0.9	0.036
18	59	0	2.0	0.053	0	2.0	0.043	0	0	0.024
14	34	2.5	3.0	0.146	3.0	3.0	0.075	1.3	1.5	0.080
7	17				3.7	3.3	0.177	1.5	0.5	0.124
17	4				0	3.8	0.061	0	0.5	0.055
Average		1.2	2.5	0.100	1.7	3.0	0.089	0.7	0.6	0.071

Cow No.	Days in lactation[a]	Time in relation to pasture feeding: 1 day after — Flavor score — No Cu added	0.1 p.p.m. Cu added	Natural copper	7 days after — Flavor score — No Cu added	0.1 p.p.m. Cu added	Natural copper	18 days after — Flavor score — No Cu added	0.1 p.p.m. Cu added	Natural copper
				(p.p.m.)			*(p.p.m.)*			*(p.p.m.)*
10	315	0	0	0.054	1.5	0	0.035	0	0	0.043
11	268	0	1.3	0.039	0.5	1.5	0.039			
16	243	0	2.5	0.048	1.5	3.5	0.039	0.5	2.5	0.033
21	240	1.3	1.5	0.025	0	2.3	0.024	0	2.5	0.026
9	174	0	1.8	0.033	0	3.8	0.036	0	3.3	0.036
15	158	0	1.0	0.021	0	2.8	0.015	0	3.3	0.014
12	124	0	0	0.047	0	0	0.029	0	1.8	0.033
13	123	0	0	0.029	0	1.0	0.025	0	1.5	0.026
19	115	0	3.5	0.040	0	3.5	0.047	0	4.0	0.022
20	77	0	1.0	0.050	0	0	0.028	0	0.5	0.032
8	73	0	0	0.031	0	1.0	0.053	0	0	0.038
Average		0.1	1.1	0.038	0.3	1.8	0.034	0.0	1.9	0.030
18	59	0	0.5	0.041	0	1.5	0.020	0.5	2.5	0.019
14	34	1.8	2.3	0.090	2.8	3.0	0.070	2.5	3.3	0.066
7	17	1.5	2.8	0.118	3.3	3.3	0.088	3.8	3.5	0.058
17	4	0	0.8	0.058	0	0	0.080	0	1.0	0.061
Average		0.8	1.6	0.077	1.5	2.0	0.064	1.7	2.6	0.051

[a] At start of pasture feeding.
[b] Samples stored five days at about 3° C.

a fairly rapid increase after the cows were returned to the dry ration. In general, the limited data for spontaneous oxidized flavor showed similar trends.

The change in feed did not influence the concentration of natural copper in the milk. The change in resistance of the milk to oxidized flavor apparently was not related to changes in the natural copper content. The only consistent trend was the gradual decrease in natural copper for the early-lactation cows.

Drenching with copper sulfate. Four cows were administered single doses of 10 g. of $CuSO_4 \cdot 5H_2O$ dissolved in about 1 liter of water. The cows had been producing milk that was classified either as spontaneous or as susceptible. In the trial with Cow No. 18 (Figure 3a), samples were taken from milks produced 12 hr. and immediately before the drench (for controls), and daily for five days thereafter. For the other three trials (Figure 3b, c, and d), samples were taken each day for five days before the drench, then twice daily for two days, and then daily for five days. The samples were scored daily for oxidized flavor and were used for determining natural copper content.

The cows varied appreciably in response to the copper sulfate drench. However, in each case the administration of copper sulfate increased the copper content of the milk, and incidence or rate of development of spontaneous oxidized flavor. Drenching Cow No. 18 caused only a slight increase in natural copper content (Figure 3a), but it was accompanied by spontaneous development of oxidized flavor for the first time in the 10 mo. of the cow's lactation. Cow No. 12 (Figure 3b) showed the greatest response to the copper sulfate drench. The

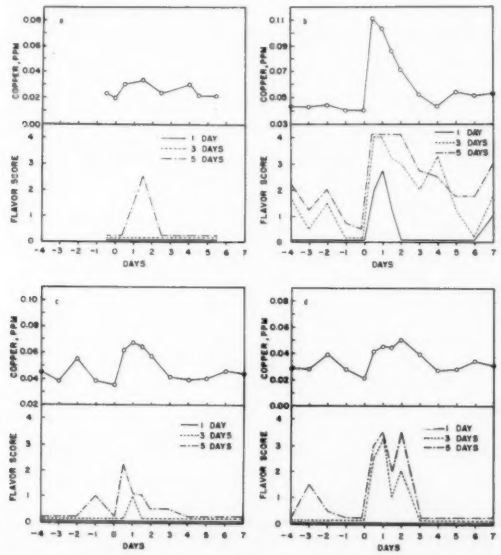

FIG. 3. Influence of a single 10-g. drench of copper sulfate on the natural copper content and development of spontaneous oxidized flavor in milk.

a—Cow No. 18, b—Cow No. 12, c—Cow No. 11, d—Cow No. 5.

natural copper content of its milk increased 0.07 p.p.m., and the milk developed spontaneous oxidized flavor as early as 12 hr. after milking. The other two cows, No. 11 and No. 5, were intermediate in response. The data also show differences among the cows in the concentration of natural copper associated with spontaneous development of oxidized flavor. The normal level for No. 11 was higher than levels for the three other cows that were accompanied by spontaneous oxidized flavor, yet its milk showed a comparatively low incidence and intensity of oxidized flavor before the drench.

These results demonstrate that large doses of copper sulfate caused an increase in the concentration of natural copper in milk (2). Furthermore, when a cow was already producing milk susceptible to oxidized flavor, the increase was accompanied by an increase in the incidence and/or rate of development of spontaneous oxidized flavor.

Correlation between natural copper and spontaneous oxidized flavor. Data accumulated during routine sampling of milk from all of the cows in the herd over a 2.5-yr. period are plotted (Figure 4) to illustrate the correlation between natural copper content and the incidence and intensity of spontaneous oxidized flavor. A number of the samples with high copper concentrations but no spontaneous oxidized flavor were produced by cows that either were on pasture or were in early lactation. Data for these samples are plotted with different symbols (squares and triangles instead of circles). These results were deleted, and the coefficient of correlation was calculated for the remaining samples. The value obtained was 0.590, which indicates that, for cows on an alfalfa hay-grain concentrate ration and not in early lactation, there is a highly significant correlation (1% level) between natural copper content and spontaneous oxidized flavor.

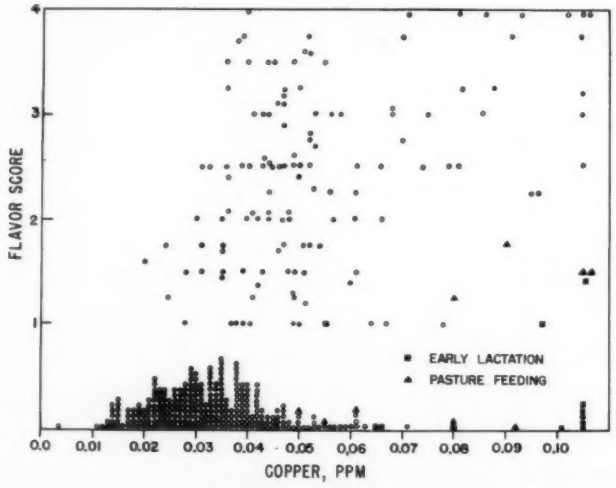

FIG. 4. Scatter diagram illustrating relation between natural copper content and development of oxidized flavor in milk produced by cows on dry feed.

These results, together with those from the experiments on drenching cows with large doses of copper sulfate, support the conclusion that when cows are maintained on an alfalfa hay–grain concentrate ration, the natural copper in milk is an important catalyst of oxidized flavor.

ACKNOWLEDGMENT

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DETERMINATION OF DEGRADATION PRODUCTS OF ASCORBIC ACID[1]

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SUMMARY

The 2,4-dinitrophenylhydrazine test for ascorbic acid was used to determine both ascorbic and gulonic acid in milk. Data are presented in support of the validity of the test and simplification of procedure. Comparisons are also made with the 2,6-dichlorophenolindophenol reduction procedure. Interpretation of the observed phenomena is made in view of other known facts found in the literature. One- to two-day-old raw milk was found to contain small quantities of gulonic acid. Immediately following pasteurization, the gulonic acid content was also found to be low, but upon storage at 40° F. it increased considerably. Analysis of nonfat dry milk failed to reveal any gulonic acid, regardless of source of the powder, and only questionable amounts were found in evaporated milk. Temperature of storage had a decided effect on the decomposition of dehydroascorbic acid and the rate of oxidation of the resulting gulonic acid in sterile milk. The oxidation of gulonic acid was generally found to proceed rapidly at elevated temperatures. Evidence is presented that exposure to sunlight and copper catalyzed the oxidation of gulonic acid, but not nearly to the same extent as elevated temperatures. The rate of formation of gulonic acid under these conditions was greater than its oxidation.

The most common procedures for the determination of ascorbic acid are based on the oxidation-reduction reaction between the reduced form of ascorbic acid and 2,6-dichlorophenolindophenol, or a coupling of 2,4-dinitrophenylhydrazine with dehydroascorbic acid, forming a derivative of *bis*-2,4-dinitrophenylhydrazone (17). The oxidation-reduction method with several modifications (2, 3, 19, 21, 22) has been used extensively in the analysis of milk.

Roe *et al.* (18) described a procedure using 2,4-dinitrophenylhydrazine by which it is possible to differentiate between the reduced and oxidized form of ascorbic acid and 2,3-diketo-l-gulonic acid, a decomposition product. As no direct analyses of gulonic acid in milk have been reported, this study was undertaken to determine its prevalence and fate on storage and processing, using the procedure of Roe *et al.* (18) with slight modifications.

TEST PROCEDURE

To 15 ml. of a 4% solution of trichloroacetic acid in a 50-ml. centrifuge tube, 5 ml. of milk was added with agitation. Two samples were necessary for a single determination. The tubes were centrifuged and the supernatant whey was decanted into a beaker containing approximately 0.75 g. Norit. After thorough mixing, the Norit suspension was filtered and 10 ml. of the clear filtrate was transferred to a test tube containing 0.1000 g. of reagent grade thiourea.

For the determination of total ascorbic and gulonic acid, 4 ml. of the solution was transferred to an Evelyn tube and 1 ml. of a 2% solution of 2,4-dinitrophenylhydrazine in 9 *N* H_2SO_4 was added. The samples were incubated at 37° C.

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for 3 hr. and subsequently cooled in an ice bath. Five milliliters of 85% H_2SO_4 was added dropwise while the tubes were still in ice water, and the contents were thoroughly mixed. The samples were warmed at room temperature for 30 min. and the per cent transmission was determined at 540 mμ in a photoelectric instrument.

For the determination of gulonic acid alone, the 10 ml. of whey containing thiourea was transferred to a large test tube, and H_2S from a cylinder was bubbled through a sintered glass gas filter for 15 min. This was followed by bubbling CO_2 for 5 min. to remove the H_2S. Four milliliters was then transferred to an Evelyn tube and the same procedure was followed as for the total ascorbic and gulonic acid.

A blank determination was performed on 4-ml. aliquots of the whey containing thiourea. Five milliliters of 85% H_2SO_4 was added while the sample was cooled in ice water, followed by 1 ml. of the 2,4-dinitrophenylhydrazine solution. From this point on, the blanks were treated in the same way as the samples being analyzed.

A calibration curve was prepared by making up solutions of ascorbic acid in concentrations ranging from 0.1 to 2.5 mg. per 100 ml. in 4% trichloroacetic acid, and proceeding the same as for total ascorbic and gulonic acid. Additional details for the preparation of reagents may be found in the literature (16, 17).

RESULTS

Table 1 shows the effect of varying amounts of Norit on the results of ascorbic acid analyses. It is apparent that an exact weight of Norit is not necessary and that a measuring spoon calibrated to deliver approximately 0.75 g. is satisfactory.

Data in Table 2 were used to determine the appropriate concentration of trichloroacetic acid for the test. As shown by Roe *et al.* (18), dehydroascorbic acid is rapidly converted to gulonic acid at a pH below 1.0. At the same time, gulonic acid in the presence of H_2S is slowly converted to unknown compounds (14), this change proceeding more rapidly with increasing pH (18). It is seen that a 4% solution of trichloroacetic acid with milk after Norit treatment yields

TABLE 1

Effect of varying amounts of Norit on the total ascorbic acid content

Product	Quantity of Norit	Total ascorbic acid
	(g.)	*(mg/100 ml.)*
Aqueous ascorbic acid solution	0.50	0.94
	0.60	0.95
	0.70	0.95
	0.80	0.93
	0.90	0.94
Pasteurized milk	0.50	1.03
	0.75	1.05
	1.00	1.05
Evaporated milk	0.50	0.28
	0.75	0.21
	1.00	0.21

TABLE 2
The pH of trichloroacetic acid–milk mixtures at different steps in the procedure

Concentration of trichloroacetic acid	First adding milk to acid	Filtrate following centrifugation	Filtrate following Norit treatment
(%)		(pH)	
10	0.77	0.68	0.83
5	0.88	0.81	1.04
4.5	0.99	0.90	1.15
4.0	1.10	1.02	1.25
3.5	1.22	1.10	1.37

a final pH of 1.25, and is at a pH higher than 1.0 at all times prior to the last step. At pH 1.25, the reduction of dehydroascorbic acid by H_2S is practically complete in 15 min. (18).

Table 3 contains analyses of total ascorbic and gulonic acid and gulonic acid alone, using a 3- and a 6-hr. incubation time. The values were read from calibration curves, also using 3- and 6-hr. incubation times. Since the calibration curves are prepared with dehydroascorbic acid, and gulonic acid couples with 2,4-dinitrophenylhydrazine at a faster rate than dehydroascorbic acid, it is not surprising to find the discrepancy in the values at different incubation

TABLE 3
Observed and calculated values for 3- and 6-hr. incubation[a]

T_3	G_3	T_6 observed	T_6 calculated	G_6 observed	G_6 calculated	T_6 observed–G_6 observed[b]	T_6 calculated–G_6 calculated[b]
			(mg/100 ml.)				
1.80	0.62	1.70	1.69	0.55	0.54	1.15	1.15
1.81	0.50	1.74	1.72	0.49	0.44	1.25	1.28
1.85	0.66	1.72	1.73	0.57	0.58	1.15	1.15
1.80	0.62	1.68	1.69	0.53	0.54	1.15	1.15
1.71	1.27	1.50	1.48	0.98	1.12	0.52	0.36
1.77	1.05	1.58	1.58	0.85	0.92	0.73	0.66
1.60	1.27	1.41	1.37	1.15	1.12	0.26	0.25
1.54	0.94	1.40	1.37	0.85	0.83	0.55	0.54
1.82	0.28	1.67	1.77	0.24	0.24	1.43	1.53
1.73	0.64	1.56	1.61	0.54	0.56	1.02	1.05
1.64	0.60	1.47	1.53	0.52	0.53	0.95	1.00
1.20	0.78	1.06	1.06	0.65	0.68	0.41	0.38
0.87	0.71	0.75	0.74	0.63	0.62	0.12	0.12
0.65	0.61	0.58	0.54	0.53	0.53	0.05	0.01
0.52	0.46	0.47	0.44	0.40	0.40	0.07	0.04
2.09	0.59	1.91	1.98	0.54	0.52	1.37	1.46
1.47	0.20	1.37	1.43	0.22	0.18	1.15	1.25
1.30	1.03	1.15	1.11	0.87	0.90	0.28	0.21
1.15	1.01	1.03	0.96	0.85	0.89	0.18	0.07
1.49	0.94	1.33	1.32	0.83	0.83	0.50	0.49
1.40	1.03	1.22	1.21	0.90	0.90	0.32	0.31
1.92	0.21	1.85	1.88	0.19	0.18	1.66	1.70
1.59	1.38	1.36	1.33	1.19	1.21	0.17	0.12
1.95	0.19	1.85	1.91	0.16	0.17	1.69	1.74
1.98	0.53	1.84	1.88	0.44	0.46	1.40	1.42
2.00	0.20	2.00	1.96	0.18	0.18	1.82	1.78

[a] T = total ascorbic and gulonic acid; G = gulonic acid; subscripts = hours of incubation.
[b] Ascorbic + dehydroascorbic acid.

times. Roe *et al.* (18) have shown, however, that with a 6-hr. incubation period the coupling rate of dehydroascorbic acid is 95% that of gulonic acid and, thus, the values are more nearly correct than those obtained with a 3-hr. incubation period.

It was felt that the 3-hr. incubation time was more convenient and, therefore, an effort was made to derive an empirical factor, by the use of which the 3-hr. incubation values could be converted to those that would be obtained after 6 hr. The expression took the form $T_6 = T_3 - FG_3$, where T is the total ascorbic and gulonic acid, F is the factor, G is gulonic acid, and the subscripts indicate the number of hours of incubation. The average factor calculated from the data in Table 3 was found to be 0.185. The determination of gulonic acid alone is not complicated by the presence of the slower-coupling dehydroascorbic acid and the G_6 value was found to follow the expression $G_6 = 0.878\ G_3$.

When no gulonic acid is present, T_6 should be equal to T_3, and the data in Table 9 support this view. Practically all observed and calculated values of T_6, as well as those of G_6, in Table 3, are within experimental error of each other.

Data in Table 4 were used to furnish a measure of the reproducibility of the test. They were collected by two technicians on 16 different samples of milk. An optimum standard deviation, δ, was calculated, using the expression

$$\delta = \sqrt{\frac{\Sigma\Sigma\ x_i^2 - n x_j^2}{n_t - n_j}}.$$

The significance of the symbols will become apparent in Table 4. The observed standard deviation of $\pm$ 0.04 mg/100 ml. indicates a fair degree of reproducibility.

To verify the recovery of known amounts of added ascorbic acid to milk and, at the same time, determine the extent to which dehydroascorbic acid is reduced to the nonreactive ascorbic acid by the hydrogen sulfide treatment, the following experiment was designed: A solution of ascorbic acid was prepared with about 0.1 g. in 100 ml. One milliliter of this solution was added to 99 ml. of milk and also to 99 ml. of water. The milk with ascorbic acid added, and the water to which the ascorbic acid was added, were then analyzed for both total ascorbic and gulonic acid and gulonic acid alone. Data are presented in Table 5.

Recovery of ascorbic acid in these four trials averaged 101.5%. In other experiments, the data of which are not presented here, recoveries of 95 to 105%, averaging fairly closely to 100%, were generally obtained. It is to be noted that within experimental error all of the dehydroascorbic acid formed from the added ascorbic acid was reduced by the hydrogen sulfide treatment.

In Table 6, individual analyses are reported on raw milk taken from farm bulk tanks 12–48 hr. after milking. These results confirm the findings of the instability of dehydroascorbic acid in raw milk (5) as reflected in a build-up of gulonic acid. No conclusions can be drawn from these data as to the relative stability of ascorbic acid in bulk tanks as compared to other means of storage.

TABLE 4

Reproducibility of the ascorbic acid determinaton

Sample No.	Total ascorbic acid	d^2 [a]
	(mg/100 ml.)	
1	1.36	0.00000
	1.36	
2	1.71	0.00045
	1.74	
3	2.00	0.00980
	2.14	
4	1.01	0.00125
	1.06	
5	1.98	0.00020
	2.00	
6	0.12	0.00045
	0.15	
7	1.30	0.000695
	1.30	
	1.33	
	1.30	
8	1.43	0.00245
	1.36	
9	0.51	0.00140
	0.47	
	0.46	
10	1.27	0.00810
	1.24	
	1.36	
11	1.17	0.000675
	1.14	
	1.17	
	1.15	
12	0.35	0.00000
	0.35	
	0.35	
13	1.26	0.000800
	1.28	
	1.30	
14	2.61	0.0004661
	2.62	
	2.59	
15	2.59	0.0060672
	2.54	
	2.65	
16	2.79	0.0032672
	2.72	
	2.72	

$d^2 = 0.0360705$

$n_t - n_j = 27$

$\delta = \pm 0.03659$

[a] d = deviation from mean.

Table 7 contains analyses of pasteurized milk over varying periods of storage. Fresh, commercial homogenized milk in paper cartons was used. Samples 3 and 4 were stored at 40° F. continuously after the initial analysis. Samples 1, 2, and 5 were removed from 40° F. storage for 20–40 min. each time an analysis was performed.

TABLE 5

Recovery of ascorbic acid and extent of dehydroascorbic acid reduction by hydrogen sulfide

Sample	Trial 1	Trial 2	Trial 3	Trial 4
	(mg/100 ml.)			
	Total ascorbic and gulonic acid			
Milk	1.19	0.98	0.46	1.80
Ascorbic Acid solution	0.97	0.97	0.96	0.96
Milk and ascorbic acid	2.22	1.99	1.36	2.78
	Gulonic acid			
Milk	0.28	0.94	0.45	0.33
Ascorbic Acid solution	0.02	0.00	0.02	0.02
Milk and ascorbic acid	0.29	0.96	0.47	0.37

TABLE 6

Ascorbic and gulonic acid in raw milk

Sample No.	AsA + DHA[a]	GA[a]	Sample No.	AsA + DHA	GA
	(mg/100 ml.)			*(mg/100 ml.)*	
1	1.40	0.44	6	1.85	0.17
2	1.82	0.18	7	1.69	0.16
3	1.89	0.23	8	1.73	0.13
4	1.71	0.19	9	1.91	0.19
5	1.82	0.08	10	1.75	0.32

[a] AsA = ascorbic acid; DHA = dehydroascorbic acid; GA = gulonic acid.

These results would indicate that the oxidation of ascorbic acid proceeds at a more rapid rate than the oxidation of gulonic acid, which increased at the expense of ascorbic acid. These data, however, are insufficient to derive the kinetics of the reactions.

Additional information concerning the stability of ascorbic and gulonic acid may be obtained from the data in Table 8. The milk was sterilized at a temperature of 300° F. in a Mallory small tube heat exchanger (7) and aseptically filled into 50-ml. screw-cap test tubes. The tubes were stored at 40 and 70° F. for a period of 79 days. A different tube of milk was used for each determination.

The ascorbic acid seems to be more stable in sterile than in pasteurized milk, presumably due to the protective action of heat-induced reducing substances, but the protection is not as long-lived as that observed in evaporated milk (2). There does not appear to be any point in storage time beyond which the ascorbic or gulonic acid content does not diminish further. After building up to a maximum in 40° F. storage, the gulonic acid disappears at a rather uniform rate. At 70° F., both ascorbic and gulonic acid are quite unstable.

Both 3- and 6-hr. incubation values for nonfat dry milk solids in Table 9 show that the ascorbic acid contents for the two incubation periods are within experimental error of each other because of the absence of gulonic acid. All of the analyses were performed on commercial products of four different manufacturers, and the history of the samples was unknown, except that some were of the instant type and others of the low or high heat type.

The fact that nonfat dry milk solids contained essentially no gulonic acid is rather difficult to explain. It would appear that gulonic acid is quite unstable

TABLE 7

Changes in ascorbic and gulonic acid during storage of pasteurized milk

Storage	Sample 1		2		3		4		5	
	AsA + DHA	GA	AsA + DHA	GA	AsA + DHA	GA	AsA + DHA	GA	AsA + DHA	GA
(days)	*(mg/100 ml.)*									
0	1.15	0.55	1.25	0.49	1.42	0.24	1.01	0.55	1.14	0.23
1	1.15	0.57	1.15	0.53	.....	.....	.....	.....	.....	.....
5	0.52	0.98	0.74	0.84	.....	.....	.....	.....	0.30	0.88
8	0.27	1.14	0.55	0.85	.....	.....	.....	.....	0.19	0.84
9	.....	.....	.....	.....	0.95	0.52	0.42	0.65	.....	.....

TABLE 8

Changes in ascorbic and gulonic acid during storage of sterile milk

Storage time	Storage at 40° F.		Storage at 70° F.	
	AsA + DHA	GA	AsA + DHA	GA
(days)	*(mg/100 ml.)*			
0 (raw)	1.75	0.32		
0	1.05	0.19		
14	0.79	0.28	0.11	0.21
21	0.17	0.58	0.00	0.00
28	0.14	0.62	0.00	0.00
35	0.06	0.52	0.00	0.00
42	0.12	0.40	0.00	0.00
51	0.02	0.27	0.05	0.00
79	0.11	0.05	0.01	0.00

TABLE 9

Ascorbic and gulonic acid in nonfat dry milk solids

Sample No.	3-Hr. incubation		6-Hr. incubation	
	AsA + DHA	GA	AsA + DHA	GA
	(mg/100 ml.)			
1	0.72	0.01	0.71	0.00
2	0.64	0.00	0.62	0.00
3	0.91	0.03	0.89	0.03
4	0.75	0.00	0.73	0.00
5	0.48	0.00	0.47	0.00
6	0.87	0.00	0.79	0.00
7	1.42	0.01	1.45	0.03
8	1.04	0.00	1.03	0.02
9	0.47	0.00	0.45	0.02

in the dry state or that its oxidation proceeds at a rate equal to or greater than the decomposition of dehydroascorbic acid. Gulonic acid has been reported in some dehydrated fruits and vegetables (11).

Table 10 contains analyses of commercial evaporated milk, the history of which was unknown. The differences between the 3- and 6-hr. incubation values were small and within experimental error of each other. However, the 6-hr. values are almost invariably higher than the 3-hr. values, a phenomenon not observed in the analysis of any of the other products. It is conceivable that evaporated milk contains substances other than ascorbic and gulonic acid which might couple with 2,4-dinitrophenylhydrazine. Some of the interfering sub-

TABLE 10

Ascorbic and gulonic acid in evaporated milk[a]

Sample No.	3-Hr. incubation			6-Hr. incubation		
	AsA + DHA + GA	GA	AsA + DHA	AsA + DHA + GA	GA	AsA + DHA
	(mg/100 ml.)					
1	0.65	0.09	0.56	0.68	0.11	0.57
2	0.57	0.16	0.41	0.61	0.16	0.45
3	0.36	0.07	0.29	0.32	0.08	0.24
4	0.34	0.02	0.32	0.35	0.04	0.31
5	0.51	0.01	0.50	0.53	0.05	0.49
6	0.32	0.04	0.28	0.34	0.06	0.28
7	0.68	0.03	0.65	0.73	0.09	0.64

[a] Reconstituted basis.

stances in the indophenol reduction test of evaporated milk found by Doan and Josephson (2) might contain dienol or ketone groups which would also interfere in the present test.

One interpretation of Table 10 would be that there is very little or no gulonic acid present and the material reported consists of interfering substances not reducible with hydrogen sulfide, which reacted to a greater extent in 6 hr. than in 3 hr. That would explain why the difference between total gulonic and ascorbic acid and gulonic acid alone was the same, regardless of whether a 3- or a 6-hr. incubation period was used. It would also substantiate the finding that 3-hr. and 6-hr. incubation values yield the same results when no gulonic acid is present.

A limited number of trials was conducted to determine the effect of sunlight and copper on the ascorbic and gulonic acid contents of milk. Table 11 shows

TABLE 11
Effect of sunlight on ascorbic and gulonic acid in milk

Time of exposure	Flavor[a]	AsA + DHA	GA	AsA + DHA + GA
(hr.)		*(mg/100 ml.)*		
0	38 Feed	1.13	0.50	1.63
½	34 Sunshine	0.50	0.83	1.33
1	31 Sunshine	0.32	0.90	1.22

[a] Scored on the basis of the American Dairy Science Association score-card.

the effect of sunlight exposure on milk in clear glass bottles for 30 min. and for 1 hr. One-day-old homogenized milk was used in the experiment.

It can be seen that nearly 72% of the ascorbic and dehydroascorbic acid was lost in an 1-hr. exposure, but only half of that amount was recovered in the form of gulonic acid. The proportion of recovered gulonic acid after the half-hour exposure is also essentially half of the lost ascorbic and dehydroascorbic acid. This would indicate that the oxidation of gulonic acid also may be catalyzed by light, but not to the same extent as that of ascorbic acid.

Table 12 shows the effect of adding 1 p.p.m. of copper, in the form of $CuSO_4$, on the changes in ascorbic and gulonic acid as a result of pasteurizing milk in 1-liter beakers immersed in a thermostatically controlled hot water bath. The milk was agitated with a glass rod and at the end of the holding period was cooled in an ice water bath.

The oxidation of gulonic acid proceeds quite rapidly during pasteurization, as compared to cold storage (Table 7), where gulonic acid diminishes rather slowly. The data in Table 12 also verify the fact that copper catalyzes the oxidation of ascorbic acid. The evidence that the oxidation of gulonic acid is also catalyzed by copper is not quite as convincing, because the quantitatives involved are rather small. However, there is a suggestion that in the presence of copper, gulonic acid diminishes somewhat faster than under the influence of heat alone. Qualitative observations not reported here indicated that the complete removal

TABLE 12

Effect of added copper on the ascorbic and gulonic acid contents of milk during pasteurization

Treatment	AsA + DHA	GA
	(mg/100 ml.)	
Raw milk	1.91	0.07
Pasteurized[a]	1.12	0.10
Pasteurized with 1 p.p.m. Cu[a]	0.07	0.19
Raw milk	1.31	0.09
Pasteurized[a]	0.97	0.13
Pasteurized with 1 p.p.m. Cu[a]	0.11	0.22
Raw milk	1.52	0.19
Pasteurized[b]	0.64	0.18
Pasteurized with 1 p.p.m. Cu[b]	0.09	0.09
Raw milk	1.95	0.08
Pasteurized[b]	0.89	0.20
Pasteurized with 1 p.p.m. Cu[b]	0.10	0.09

[a] 143° F. for 30 min.
[b] 145° F. for 60 min.

of ascorbic and gulonic acid required heating in the presence of 1 p.p.m. of copper at 145° F., with agitation provided by bubbling oxygen through milk for a period of little over an hour.

Further information as to the effectiveness of copper as a catalyst for the oxidation of gulonic acid may be obtained from an experiment in which a sample of pasteurized milk was treated with 1 p.p.m. of copper and stored at 40° F. for four days. This sample was intended for a milk blank in the indophenol reduction test for ascorbic acid. An analysis revealed that it contained 0.17 mg. per 100 ml. of dehydroascorbic acid and 1.10 mg. per 100 ml. of gulonic acid. Thus, in cold storage the oxidation of gulonic acid is slow, even in the presence of copper.

Table 13 contains a comparison of analyses on raw and pasteurized milk performed by the 2,4-dinitrophenylhydrazine procedure and the indophenol reduction method. The indophenol titration was performed on milk serum prepared by treatment with 6% trichloroacetic acid. Only reduced ascorbic acid was determined by the reduction procedure.

TABLE 13

Comparison of values obtained by 2,4-dinitrophenylhydrazine and 2,6-dichlorophenolindophenol procedures

Sample	AsA + DHA by dinitrophenylhydrazine procedure	AsA by indophenol titration
Pasteurized milk	1.17	1.12
	1.33	1.17
	1.38	1.31
	1.08	0.98
Raw milk	1.71	1.21
	1.96	1.52
	1.38	0.77
	1.82	1.35
	1.89	1.32

As would be expected, the two methods gave values which agreed more closely in pasteurized milk than in raw milk. It has been shown (22) that in freshly pasteurized milk practically all of the ascorbic acid is in the reduced form. In raw milk, on the other hand, there may be a considerable amount of dehydroascorbic acid (5) which would not be detected by the indophenol titration. The difference in the values obtained by the two tests, of the order of 0.5 mg. per 100 ml., is a reasonable value for dehydroascorbic acid in one- to two-day-old raw milk. Values of that magnitude were reported by Hand (5).

DISCUSSION

The fact that 2,3-diketo-1-gulonic acid interferes in the test of ascorbic acid by the procedure of Roe *et al.* (12, 17) was shown by Penney and Zilva (15). They also established the difference in the rate of reaction for the two compounds, and proposed a scheme for differentiating between them, based on their reaction rate (13). Roe *et al.* (18) found that with an incubation period of 6 hr. at 37° C. the coupling of dehydroascorbic acid is 95% that of gulonic acid and that, after 14 hr., they are practically the same. They chose the 6-hr. incubation period to eliminate the possibility of reduced ascorbic acid reacting with the hydrazine which is known to occur after 8 hr. of incubation. The only justification for the 3-hr. incubation period suggested in this study was the time saved and the fact that in milk the application of the correction factor resulted in values which, within the limits of experimental error, were indistinguishable from the values obtained with a 6-hr. incubation period.

Penney and Zilva (13) postulated that 2,4-dinitrophenylhydrazine couples only with gulonic acid. Thus, dehydroascorbic acid would first have to be converted to gulonic acid before coupling could occur.

Lactose did not appear to interfere in the test in this study. Five per cent lactose solutions, both with and without the hydrogen sulfide treatment, gave values equal to the reagent blank. The fact that some of the analyses on milk reported here failed to demonstrate the presence of any ascorbic or gulonic acid further supports the view that lactose did not interfere. In fact, it presents good evidence as to the specificity of the method, although no data are presented here to offer conclusive proof. Chapman *et al.* (1) have shown that there is no interference due to iron and copper ions.

In the performance of this test, ascorbic acid is oxidized to dehydroascorbic acid with Norit. If the Norit treatment were eliminated, it should be possible to differentiate between dehydroascorbic acid and ascorbic acid. A number of attempts to accomplish this was made in the present study, but with variable and inconclusive results. Four per cent trichloroacetic acid was found inadequate to yield a clear milk whey in the absence of the Norit treatment, and the results were generally higher than would be predicted, in some cases exceeding the total ascorbic and gulonic acid content. It was concluded, therefore, that the Norit treatment was an essential step in the performance of the test, in that it removed certain pigments and possibly other substances which subse-

quently would interfere in the test. It is for this reason that the Norit treatment was used in the determination of gulonic acid alone. Since reduction with hydrogen sulfide would be necessary in any case, the procedure is not unduly complicated.

Some question may arise as to the completeness of reduction of dehydroascorbic acid in trichloroacetic acid solution of pH 1.2. Levenson *et al.* (9) found that complete reduction occurred at a pH of about 3.5 and that at lower pH levels less than complete recovery was obtained. Their investigations were not carried out in trichloroacetic acid solution. Roe *et al.* (18) obtained nearly complete reduction at pH 1.2 in 5% HPO_3. Spruyt and Donath (20) stated that reduction may be carried out with hydrogen sulfide in every concentration between 0–20% trichloroacetic acid. Other investigators (10), however, found that completeness of reduction is dependent on the pH of the medium. The chief cause of difficulty in obtaining complete reduction appears to lie in the irreversible decomposition of dehydroascorbic acid.

An examination of Table 5 discloses that the reduction of dehydroascorbic acid under the present experimental conditions is nearly complete, as the quantities of gulonic acid found in milk are within the limits of experimental error, the same as those in the same milk to which a known quantity of ascorbic acid was added. If an assumption were made that the reduction is not complete, the quantity of gulonic acid found would be high. The cases, then, in which no gulonic acid was found (Table 9) would be difficult to interpret, unless one could establish that the degradation of ascorbic acid does not proceed through the gulonic acid step.

The data presented here indicate that gulonic acid is not the last step in the degradation of ascorbic acid in milk. It was shown that it diminishes during pasteurization and storage and possibly under the influence of sunlight and copper. The degradation of gulonic acid is believed to be oxidative in nature, resulting in the formation of oxalic acid and l-threonic acid (6). The presence of these substances in milk has not been substantiated, but from the knowledge of the chemistry of ascorbic acid, they would be expected to be present in amounts varying with the conditions under which the milk was handled and processed.

Guthrie and Krukovsky (4, 8) made the interesting observation that milk was protected against oxidized flavor when processed in such a way as to destroy quickly all the reduced ascorbic acid. Their findings were confirmed here, but it was noted that practically all gulonic acid is also destroyed before this protection is obtained. With the gulonic acid gone, oxidized flavor did not develop for 15 days (the length of the experiment), and for five to seven days in samples to which 1 p.p.m. copper was added. Controls processed in the conventional manner, either with or without copper added, developed an oxidized flavor within 24 hr.

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A VOLUMETRIC METHOD FOR ESTIMATING THE UNDENATURED SERUM PROTEIN IN MILK[1]

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SUMMARY

A rapid and simple method for determining the undenatured serum protein in milk, based on measurement of the volume of precipitated serum protein, is described. It involves the acid precipitation of the casein and the denatured serum protein and their removal by filtration. The soluble undenatured whey proteins in 7 ml. of filtrate are precipitated with 3 ml. of 10% phosphotungstic acid. Upon controlled centrifugation, the volume of the precipitate is obtained and converted to milligrams of whey protein nitrogen per gram of nonfat milk solids by means of a standard curve or the volume of precipitate may be used directly as the unit of measurement. Thus, the suitability of milk for a specific purpose is indicated.

The relation between milliliters of precipitate and milligrams of undenatured whey protein nitrogen as determined by Kjeldahl analysis has a correlation coefficient of 0.97. A standard curve is given which can be used routinely with the indicated equipment.

For some uses of milk, it is necessary to know the quantity of undenatured serum protein in the milk. Serum proteins are partially denatured during the manufacture of dry milk, particularly during the forewarming period. When nonfat dry milk (NFDM) is used in making cottage cheese, the amount of denaturation must be small if normal shrinkage of the curd and expulsion of whey are to take place. For bread-making, high-heat treatment of the milk with greater serum protein denaturation is desired, since it leads to improved baking quality. Yet, serum protein denaturation, in itself, has never been shown conclusively to be the change that improves the baking quality of the milk, and it is possible that some parallel change is responsible for the improvement (4).

There is no sharp division in undenatured whey protein nitrogen (UWPN) values to differentiate good and poor baking quality in milk. A suggested maximum value[1] for high-heat nonfat dry milk is 1.5 mg. per gram of nonfat solids as determined by the Harland and Ashworth procedure (1). A low-heat product suitable for cottage cheese manufacture should have a minimum value of 6.0 mg.

The method described in this paper is based on the measurement of the volume of undenatured serum protein precipitated by the addition of a suitable protein precipitating agent to a casein-free filtrate of milk. The range in volume of precipitate from a high-heat to a low-heat sample is shown in Figure 1.

MATERIALS AND EQUIPMENT

Reagents:

1. 10% acetic acid in distilled water: 10 ml. glacial acetic acid to 90 ml. water.

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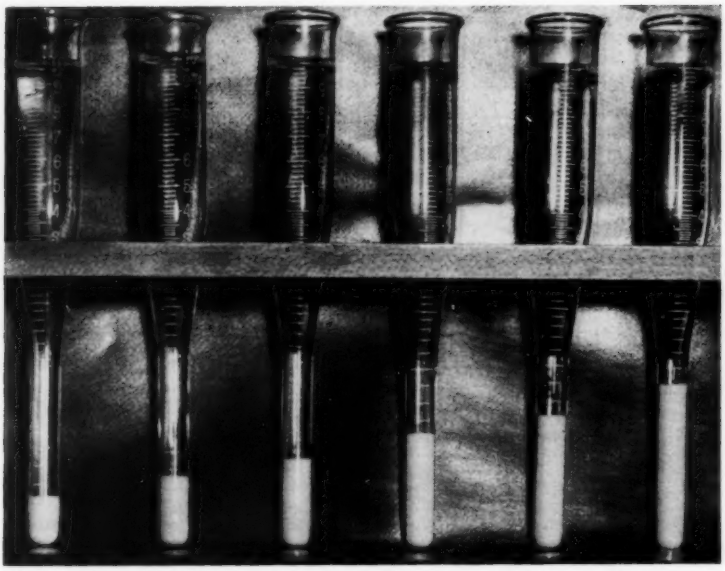

FIG. 1. The range in volume of precipitate from a high-heat to a low-heat sample.

2. 10% phosphotungstic acid in distilled water: 10 g. of phosphotungstic acid is diluted to 100 ml. Mild heat may be necessary to get all the phosphotungstic acid into solution.

Equipment:

1. Size 2 International centrifuge with a No. 240 head and No. 303 cups.
2. Tachometer for the centrifuge.
3. 100-ml. burette.
4. 300-ml. Erleneyer flasks.
5. Wrist-action shaker.
6. 10-ml. Kolmer centrifuge tubes.
7. 70-ml. delivery pipettes.
8. 100-mm. funnels.

EXPERIMENTAL PROCEDURE

1. A 10-g. representative sample of NFDM is weighed accurately and transferred to a 300-ml. Erlenmeyer flask. For dry whole milk the nonfat solids should equal those in 10 g. of NFDM. Ninety-three ml. of distilled water are added from a 100-ml. burette.
2. The flasks are attached by their necks to a wrist-action shaking machine and agitated for 10 min. at moderate speed. If a shaking machine is not available, samples should be mixed by swirling for 10 min.

3. Seven milliliters of a 10% solution of acetic acid are added slowly to the reconstituted skimmilk or milk with swirling of the flask. It is important that the swirling be not so forceful that the curd is broken and filtration is made more difficult. When acid is being added to the milk, mixing is necessary to distribute the acid uniformly, but harsh mixing produces a curd so fine that filtration is more difficult.
4. The precipitate should not be allowed to stand for more than 3 min.
5. The precipitate is removed by filtration, using a No. 3 Whatman filter paper. If the filtrate is not free of precipitate, it should be refiltered.
6. Seven milliliters of filtrate are pipetted into a 10-ml. Kolmer centrifuge tube.
7. Three milliliters of 10% phosphotungistic acid are added.
8. The suspension thus formed is mixed with a glass stirring rod until the particles are finer and will produce more uniform packing. Nonuniform packing is a potential source of error in the production of good replicates.
9. The tubes are centrifuged immediately in the following manner:

   The tachometer is lowered and the clock set for 25 min. The centrifuge is started and a speed of 500 r.p.m. is attained in the first 15 sec. In the second 15 sec. a speed of 1,000 r.p.m. is attained, and so on to 2,000 r.p.m. Then, the speed is maintained at 2,000 r.p.m. It is important that the centrifuge be allowed to come to a stop slowly.
10. The volume of precipitate is measured to a tenth of a milliliter and estimated to the nearest hundredth from the calibrations on the centrifuge tube.

PREPARATION OF THE STANDARD CURVE

To obtain a standard curve of maximum accuracy, undenatured whey protein nitrogen determinations were made by the Kjeldahl method on separate milk samples that had received different heat treatments. To do this, 30-g. samples of NFDM were reconstituted to a 10% solution with water, and 10% acetic acid was added to obtain a pH of 4.65. The necessity of using more concentrated solutions, in order to have enough precipitate for accurate determination of the volume of the whey protein, precluded the use of the dilute solutions of acetic acid (.017 M) and sodium acetate (.01 M) employed by Rowland (5). The buffering action of the milk produced the correct pH without use of sodium acetate. The casein-free filtrate was obtained by filtering through a double Whatman No. 1 filter paper. The soluble undenatured whey proteins were precipitated by adding one part 40% trichloroacetic acid to one part of milk (6). Kjeldahl nitrogen determinations were made on the noncasein and the nonprotein filtrates, using the catalysts employed by Rowland. The nonprotein nitrogen values were subtracted from the noncasein nitrogen values to obtain the UWPN. The milligrams of UWPN/g of NFDM are plotted against the milliliters of precipitate in Figure 2, and the ratio of unknown to control in Figure 3.

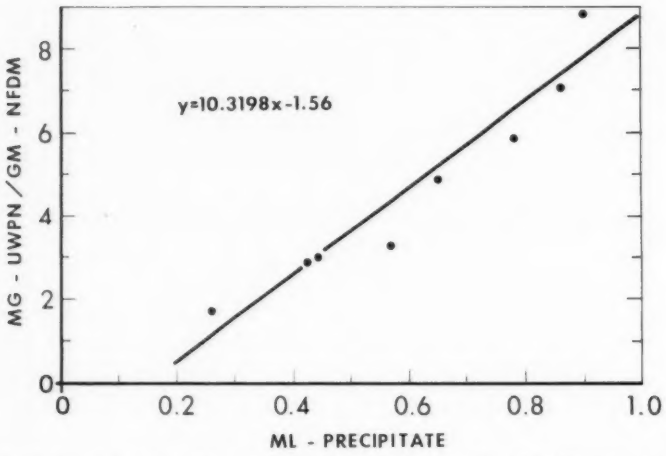

FIG. 2. Relation of the milligrams of undenatured whey protein nitrogen per gram of NFDM to the milliliters of precipitate.

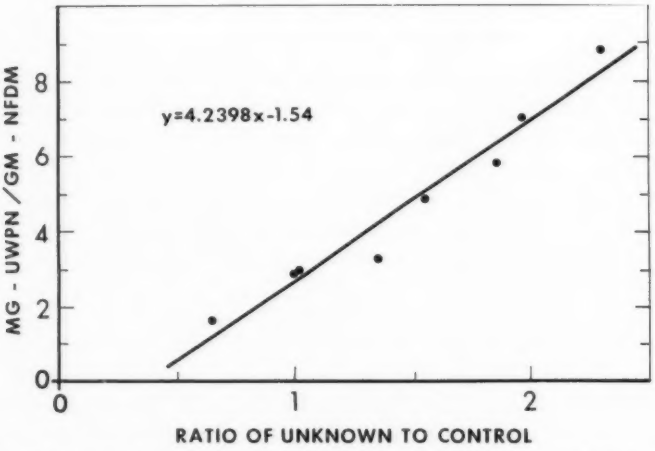

FIG. 3. Relation of milligrams of undenatured whey protein nitrogen per gram of NFDM to milliliters of precipitate using a control.

EXPERIMENTAL RESULTS

Tables 1 and 2 give typical results with milks of various heat treatments. Table 1 shows the correlation coefficient between mg. UWPN/g of NFDM (Column 2) as determined by Kjeldahl analysis, and milliliters of precipitate (Column 3) is + 0.97. The standard error for the determination of the values appearing in Column 3 is 0.01 ml. Table 2 shows the standard errors of four samples done in triplicate. Figure 2 shows the standard curve that was obtained by plotting the values in Columns 2 and 3 of Table 1. Each value represents the mean of eight determinations.

Sometimes it is more satisfactory to use the ratio of the milliliters of precipitate from a control milk to the milliliters of precipitate of the unknown. A reconstituted milk has relatively good keeping quality and so the same control can be used repeatedly. The reason for the ratio method of expressing the results is that the volume of the precipitate of the unknown varies as the control. This method of calculation gives a correlation coefficient of + 0.98 and a standard error of 0.03 between the ratio and the milligrams of protein nitrogen found in the casein-free filtrate by Kjeldahl analysis. For the control, the samples called "C (high-heat)" were used. Column 4 of Table 1 represents the ratio of the milliliters of precipitate of the control (the control had the same preparation as the unknown) divided into the milliliters of precipitate of the unknown. Each value represents the mean of four separate determinations. Values in Columns 2 and 4 of Table 1 are plotted in Figure 3.

It was found that fat did not interfere with the results as long as the calculations were based upon the measurement of the volume of precipitate from a casein-free filtrate. Therefore, if the weight of the samples of fluid or dry whole milk is based upon the weight of the nonfat portion, the results are comparable. This procedure is satisfactory for fluid whole and skimmilk and for whole and skimmilk powders.

DISCUSSION OF RESULTS AND COMPARISON WITH THE HARLAND AND ASHWORTH PROCEDURE

The present method, in which the volume of a precipitate is used to measure undenatured serum proteins, is based upon acid precipitation of casein at its isoelectric point, pH 4.60–4.70. The correct pH is obtained upon addition of 7 ml. of 10% acetic acid to 93 ml. of reconstituted skimmilk or fluid milk that contains the nonfat solids in 10 g. of NFDM. The Harland and Ashworth procedure is based upon the salting out of the casein and denatured whey proteins and their removal by filtration. The whey proteins remaining are precipitated with 10% HCl and the resulting turbidity is measured by means of a photoelectric cell. The per cent transmission is converted to milligrams of undenatured whey protein nitrogen by use of a standard curve.

The major difference in protein precipitants is that they precipitate different amounts and/or fractions of UWPN. This is shown in Tables 2 and 3. Table 2 shows the difference by Kjeldahl analysis between 10% phosphotungstic acid and 40% trichloroacetic acid in the precipitation of whey proteins. The 10% phos-

TABLE 1

Comparison of the milligrams of undenatured whey protein N/g of nonfat dry milk as determined by the Rowland method and the milliliters of precipitate of UWPN by the volumetric method

Sample No.	UWPN/g NFDM	Precipitate	Ratio to the volume from the control
	(mg.)	*(mg.)*	
6	1.65[a]	.26[b]	0.65[c]
C (High-heat)	2.87	.42	1.00
B5	3.00	.44	1.02
C4	3.30	.57	1.36
D5	4.86	.65	1.55
C2	5.84	.78	1.86
C1	7.06	.86	1.97
1	8.80	.90	2.30
Standard error		.01[d]	.03[d]

[a] Mean of duplicates.
[b] Mean of eight determinations.
[c] Mean of four determinations.
[d] Standard error of all determinations in column.

TABLE 2

Comparison of trichloroacetic acid and phosphotungstic acid as precipitants of UWPN

Precipitate	Standard error	UWPN/g NFDM by Kjeldahl Method		
(ml.)		*(mg.)*		
10% Phosphotungstic Acid		10% Phosphotungstic Acid	40% Trichloroacetic Acid	Difference
0.35[a]	.017	3.18[b]	2.76[b]	0.42
0.44	.012	3.71	3.27	0.44
0.67	.025	6.09	5.61	0.48
0.85	.018	8.73	8.43	0.30

[a] Mean of triplicates.
[b] Mean of duplicates.

TABLE 3

Comparison of results by the volumetric and the Harland and Ashworth methods on dry whole milk (DWM) and nonfat dry milk (NFDM)

Sample No.	Volumetric		Harland and Ashworth	Difference
	Precipitate	UWPN/g NFDM	UWPN/g NFDM	UWPN/g NFDM
	(ml.)	*(mg.)*	*(mg.)*	*(mg.)*
NFDM 1	1.00[a]	8.9	7.5[a]	1.4
NFDM 2	0.99	8.8	7.3	1.5
DWM 1	0.88	7.6	6.2	1.4
DWM 2	0.88	7.6	6.0	1.6
NFDM C	0.88	7.6	5.1	2.5
NFDM P	0.84	7.2	5.3	1.9
NFDM S	0.70	5.7	3.4	2.3
NFDM ADMI	0.40	2.6	1.3	1.3
NFDM 3	.34	2.0	1.2	0.8
DWM 3	.32	1.1	1.0	0.1

[a] Mean of duplicates.

photungstic acid precipitated more whey protein than the 40% trichloroacetic acid. Jenness (3), investigating the difference between salt precipitation (Harland and Ashworth) and hydrochloric acid precipitation (Rowland), found that salt precipitated a fraction that the acid did not, and that this fraction contained a large proportion of at least one of the heat-stable proteins formerly classified as proteose-peptone proteins.

Table 3 shows the difference between the volumetric and the Harland and Ashworth methods when milliliters of precipitate were converted by the standard curve to UWPN/g NFDM. There is too much variability between the two methods to establish an accurate conversion factor. This is particularly true of low-heat products (2).

An advantage of the volumetric method is that the volume (milliliters) of precipitate can serve directly as the unit of measurement, to indicate the relative amount of UWPN in NFDM. This eliminates any possible error resulting from an assumption that all undenatured proteins in the whey have the same nitrogen content.

The suggested maximum milligrams of UWPN/g of NFDM for good baking quality is 1.5 This corresponds to 0.30 ml. of precipitate. The suggested minimum for cottage cheese is 6.0 mg. This is equivalent to 0.73 ml.

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STUDIES ON THE OCCURRENCE, ORIGIN, AND SIGNIFICANCE OF AMMONIA IN EVAPORATED MILK [1]

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SUMMARY

The ammonia content of evaporated milk was found to increase with increasing heat treatment. Trials with dialyzed and bisulfite-treated milks have shown that ammonia production is independent of browning reaction. It was found that, of the total ammonia in milk heated at 242° F. for 15 min., 44% comes from urea, 26% from casein, and 23% from the whey proteins; the remaining 7% is attributed to dialyzable peptides and amino acids.

Ammonia occurs naturally in milk, as a component of the nonprotein nitrogen group, but it may result from microbial action, also. In this respect, Kluge (3) and Polonovski (5) have advocated the direct measurement of ammonia, for assessment of the quality of dairy products, especially fluid milk, when titratable acidity has been fraudulently neutralized.

There are reports (3, 5, 7) that ammonia content increases when milk is heated. No information is available, however, on the precursors of heat-generated ammonia and its relation to the browning reaction.

The present work was directed toward determining the extent and source of heat-generated ammonia, and studying its significance to the browning reaction.

MATERIALS AND METHODS

Evaporated milk was obtained from a commercial condensery. Samples were removed at random from the conveyor before sterilization, transported to the laboratory in ice water, and experimental heat treatments completed the same day. Heat treatments were conducted in a Fort Wayne pilot batch sterilizer, with the reel continuously operating in the following manner: *(a)* Temperature raised to 220° F. as fast as possible (6 min.); *(b)* temperature raised from 220 to 242° F. at the rate of 3° F. per minute; *(c)* temperature maintained at 242 ± 1° F. for desired periods (e.g., 15 min.), then cooled 15 min. in tap water in the sterilizer.

Quantitative recovery of ammonia from evaporated milk. Milk at room temperature and in cans was thoroughly agitated and the top cut open. A 50-ml. portion was placed in a 500-ml. beaker, and 200 ml. of 15% trichloroacetic acid was added. The curd was dispersed with a glass rod, and the whey allowed to separate by standing for about 15 min. The supernatant was filtered through Whatman No. 1 paper and the filtrate collected in a 400-ml. Erlenmeyer flask. A 100-ml. portion of double-distilled water was added to the curd, which was

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redispersed with a glass rod. This material, divided into two equal portions, was centrifuged 5 min. at 2,300 r.p.m. in large centrifuge tubes, in a centrifuge with a peripheral diameter of 38 cm.

The supernatant was filtered through a new filter paper and collected in the same Erlenmeyer flask. The ammonia was removed by placing a 70-ml. portion of whey in a gas scrubbing tower and adding 5.0 ml. of 33% NaOH. This brought the pH to about 10.5. To another scrubbing tower, 50 ml. of 2% boric acid was added, and the two towers were connected with a short piece of tygon tubing. Nitrogen (commercial grade) was passed through concentrated sulfuric acid, and bubbled through the alkaline whey and thence to the boric acid trap.

Vigorous bubbling for 4.5 hr. gave essentially complete recovery (Figure 1). Afterwards, a 2.0-ml. portion of the boric acid from the trap was placed in a test tube, 0.5 ml. of Nessler's reagent (2) added, the tube well shaken, and 5.0 ml. of double-distilled water added. The blank was prepared by mixing 2.0 ml. of 2% boric acid, 0.5 ml. of Nessler's reagent, and 5.0 ml. of water in another test tube.

The samples were measured against the blanks in a Beckman DU spectrophotometer at 450 mμ. The values of the concentrations were obtained by reference to a previously prepared calibration curve.

Quantitative recovery of ammonia from model systems. In the model systems used, e.g., urea in phosphate buffer, the steps necessary for removing protein

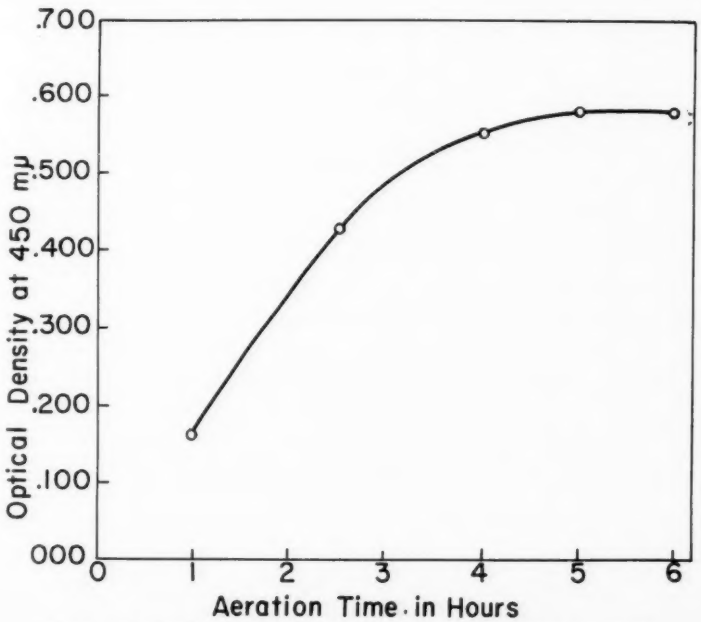

FIG. 1. Relationship between time of aeration and ammonia recovered.

material were eliminated from the procedure. Seventy milliliters of the aqueous material was placed in the gas scrubbing tower, and 5.0 ml. of 33% NaOH added. To the other tower, a 50-ml. portion of 2% boric acid was added; the remainder of the procedure was the same as for evaporated milk.

Reflectance measurements. The reflectance of the milk was measured at 400 mμ with a Beckman DU spectrophotometer equipped with reflectance attachment (1).

RESULTS AND DISCUSSION

To study any relationship between ammonia production and degree of browning, a series of samples was subjected to various heat treatments (different times at 242° F.). The data in Figure 2 indicate that the two phenomena occur simultaneously, during both heat processing and storage at room temperature for at least 6 mo. However, experiments with bisulfite-treated milk (Table 1) and dialyzed milk (Table 2) show that the two phenomena are independent of each other.

It can further be concluded that the precursors of ammonia are, in part, dialyzable, for much less is realized after dialysis (9.2 compared with 4.2 p.p.m.). The dialyzed sample to which lactose was added before sterilization showed a reflectance value about equal to that expected for normal evaporated milk. This

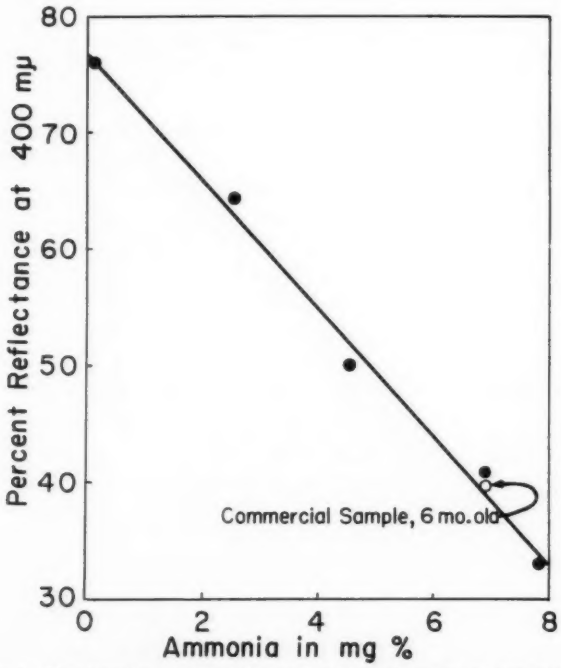

FIG. 2. Simultaneity of ammonia production and extent of browning.

TABLE 1
Ammonia production and browning in bisulfite-treated milk

5% Bisulfite added	Degree of browning	Ammonia
(ml. per can)	*(% reflectance at 400 mμ)*	*(p.p.m.)*
0 (Control)	54.5	9.2
0.5	59.5	9.3
0.7	59.5	10.0

TABLE 2
Ammonia production and browning in dialyzed milk [a]

Description of sample	Degree of browning	Ammonia
	(% reflectance at 400 mμ)	*(p.p.m.)*
Dialyzed milk, lactose added[b] before sterilization	49	4.2
Dialyzed milk, lactose added[b] after sterilization	72	4.2

[a] 1.5 liters of evaporated milk were dialyzed against a phosphate buffer (pH 6.5) made by dissolving 47.0 g. Na_2HPO_4 and 67.5 g. KH_2PO_4 in 11.5 liters of distilled water. Time of refrigerated dialysis, 29 hr.; total dialyzable solids removed, 80%.

[b] 35 g. of lactose were added to each can of dialyzed milk.

indicates that the nonlactose-dialyzable materials play a minor role in the browning reaction of evaporated milk.

An experiment performed[2] with whole milk, rennet whey, and dialyzed milk[3] sterilized at 242° F. for 15 min. indicated that 26% of the ammonia originated from the casein and 52% from dialyzable precursors (Table 3).

The most abundant nonprotein nitrogenous substances of milk are urea (6), creatine (6), and free amino acids (4). A series of trials was performed with

TABLE 3
Casein and nonprotein nitrogenous compounds as precursors of ammonia

Description	Ammonia produced	Conclusions
	(p.p.m.)	
Whole milk	7.8	2.0 p.p.m. from casein (26%)
Rennet whey	5.8	
Dialyzed milk	3.8	4.0 p.p.m. from dialyzable materials (52%)

model systems in a pH 6.6 phosphate buffer, to determine the relative heat lability of these compounds with respect to generation of ammonia. The materials used were employed in the concentrations that occur in evaporated milk, and the solutions were placed in evaporated milk cans and sterilized at 242° F. for 15 min. The results are shown in Table 4.

These data show that urea is the most important ammonia producer within the nonprotein nitrogenous group, that the role played by creatine is negligible, and that methionine also is a precursor of ammonia. Both the intensity of methional odor and the amount of ammonia generated were considerably greater

[2] Experiment done with pasteurized, homogenized milk.

[3] Same procedure as described earlier. Buffer replaced after 24 hr. and dialysis carried out for another 24 hr.

TABLE 4

Relative heat lability of nonprotein nitrogenous compounds

System	Ammonia produced	Observations
	(p.p.m.)	
Urea (unheated)	0	
Urea	11.6	
Urea + lactose[a]	9.2	
Creatine	0.2	
Creatine + lactose	0.2	
Methionine	0	Slight methional odor
Methionine + ascorbic acid	2.2	Strong methional odor

[a] The pH after sterilization was 5.4.

in the presence of ascorbic acid, indicating that the amino acid probably undergoes the Strecker degradation when heated at 242° F. for 15 min.

Less ammonia was produced from urea in the presence of lactose than from urea alone. This might be explained in terms of the lowering of pH that accompanies the browning reaction, since the production of ammonia from urea depends on pH (Table 5).

TABLE 5

Effect of pH on pyrolysis of urea

Buffer ratio	pH after addition of urea	Ammonia produced
		(p.p.m.)
$\frac{M/15\ Na_2HPO_4}{M/15\ KH_2PO_4}$		
2 : 8	6.15	8.8
0.5 : 9.5	5.45	7.7
0.25 : 9.75	5.07	6.6

Trials with homogenized pasteurized, extensively dialyzed,[4] and urea-fortified dialyzed milks, indicate the importance of urea as an ammonia precursor (Table 6).

From the information in Tables 3 and 6, it is possible to obtain values for the relative contribution of each precursor to the over-all level of ammonia. The

TABLE 6

Role of urea in production of ammonia

System	Ammonia produced	
	(p.p.m.)	
Whole milk	8.2	
Dialyzed milk	3.8	
Dialyzed milk + urea	7.4	3.6 due to urea (44%)

values for casein (26%) and urea (44%) are given directly. A value of 23% can be calculated for the whey proteins. The remaining 7% is attributed to dialyzable peptides and amino acids. Creatine produces an insignificant amount

[4] Milk dialyzed for 38 hr., according to the procedure described earlier.

of ammonia when heated in a model system. The other minor nitrogenous constituents occur in extremely low concentrations and, consequently, are of little significance.

Although ammonia has a typical odor, its role as a positive organoleptic component in milk never has been established, probably because of the masking effect of other compounds. However, because the pH of evaporated milk ranges from 6.2 to 6.5, most of the ammonia is present in the volatile form and probably plays a not unimportant role—either as a primary flavor component or one with a pronounced synergistic effect.

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COUNTERCURRENT DISTRIBUTION OF MILK FAT TRIGLYCERIDES [1]

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SUMMARY

The purpose of this study was to investigate the application of countercurrent distribution to the separation of milk fat triglycerides. Of the solvent systems tested, the most satisfactory was a 12:7:5 mixture of pentane, 2-nitropropane, and nitromethane. The distributions were followed gravimetrically and by determination of iodine values and saponification equivalents of selected fractions or groups of fractions. The fractionation was influenced by degree of unsaturation and molecular weight of the triglycerides. Although useful separations were obtained with 100 stages, better resolution was achieved by applying the technique to samples previously separated from milk fat by crystallization from pentane at low temperatures. The +4° C. precipitate and −53° C. filtrate were fractionated by countercurrent distribution, and the fatty acids in selected fractions were determined by ultraviolet spectrophotometry and gas-liquid chromatography. Evidence was obtained that there is some degree of selectivity of fatty acids during the formation of milk fat. From the analysis of the +4° C. precipitate, the evidence seems clear-cut that a small amount of tripalmitin is present in addition to dipalmitostearin and dipalmitomyristin. In the −53° C. filtrate, the monoethenoid acids are associated with the higher molecular weight saturated acids, whereas the polyethenoid acids are associated with the lower molecular weight saturated acids.

Much less is known about milk triglycerides than about the fatty acids of milk fat (19, 24). Separating individual glycerides from natural fats is difficult, especially so from milk fat because it contains a relatively large number of different fatty acids and of theoretically possible glycerides. Several investigators (1, 3, 4, 6, 13, 18) have used fractional crystallization, attempting to isolate less complex glyceride mixtures from milk fat. Amberger (3, 4) isolated small amounts of palmitodistearin, stearodipalmitin, butyrodiolein, butyropalmitoolein, and oleodipalmitin, but found only traces of the simple triglyceride triolein. Achaya and Hilditch (1) calculated the amounts of possible component glycerides in eight milk fat fractions and found no evidence of the presence of tributyrin or triolein. Difficulties in the interpretation of analytical data obtained from such fractions have resulted in divergent views (19) concerning the types of triglycerides and distribution pattern of fatty acids in milk fat.

In 1944, Craig (7) introduced the technique of countercurrent distribution and, since then, this method has been used widely to isolate solutes, including lipids, which are otherwise difficult to separate. Separation of solutes is effected on the basis of differences in their solubilities in two immiscible solvents. The partition coefficient (K) of a given solute in a given solvent system is a characteristic constant. K is defined by the equation $K = \frac{C_1}{C_2}$, where C_1 and C_2 are,

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[1] The data in this paper are taken from a thesis presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of California.

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respectively, the concentrations of the solute in the lighter, upper-phase and the heavier, lower-phase solvent layers. In the apparatus designed by Craig and Post (9), the heavier phase is contained in a series of glass cells. After placing the solute in the first cell, the lower phase is mixed and allowed to equilibrate with an equal volume of upper phase. When the phases separate, the upper phase is transferred to the second cell. A fresh upper phase is added to the first cell, and the phases in both cells are mixed again and allowed to separate. The process is repeated to move successive upper phases across the lower phases countercurrently. A solute that is much more soluble in the upper phase will pass across the train of cells at the forefront of the upper phases. If the solute is much more soluble in the lower phase, it will remain in the first cells of the train. If both phases attract the solute to exactly the same degree, the solute will move to a halfway position in the train of cells. The number of cells required to separate a mixture of solutes depends on the closeness of the solute partition coefficients and the efficiency of the solvent system.

Dutton (10), Ahrens (2), and Olley (21) have reviewed the application of countercurrent distribution to the study of lipids. The technique has been successfully applied to fractionating the triglycerides of linseed oil (11) and soybean oil (12, 23).

Countercurrent fractionation of milk fat triglycerides was explored in the present investigation. Techniques are discussed and the results in terms of the fatty acid and glyceride composition of selected fractions are given.

EXPERIMENTAL PROCEDURE

Materials. Milk fat was obtained in September, 1956, from cream separated from the pooled milk of Holstein, Jersey, and Ayrshire cows. After the cream was churned, the butter granules were washed with distilled water and melted at 50° C. After the foam was removed from the surface, the liquid fat was filtered at 50° C. in small lots to obtain a clear, dry product. Part of this fat was fractionated by crystallization from pentane at +4, −13, −23, and −53° C., using a freezing bath and filtering device (26) and method (14) previously described. The original fat, the +4° C. precipitate, and the −53° C. filtrate were studied.

Apparatus. A countercurrent distribution apparatus[3] developed by Craig *et al.* (8) and equipped with automatic filling device and driving mechanism was employed for the fractionations. The train of tubes consisted of 100 glass cells, each holding 40 ml. lower phase and 40 ml. upper phase.

Methods of analysis. The countercurrent distributions were followed gravimetrically. Most of the solvent was removed under reduced pressure (2 mm. mercury) from each fraction in a rotating round-bottom flask attached to a Rinco evaporator.[4] Then the fractions were transferred to tared 50-ml. Erlenmeyer flasks, the solvent was removed completely with the evaporator, and the residue was weighed. The maximum temperature that the samples were exposed

[3] H. O. Post Scientific Instrument Co., Maspeth, New York.

[4] A. S. Aloe Co., San Francisco, Calif.

to was 70° C., for not more than 20 min. All samples were stored at −21° C. under nitrogen and analyzed as soon as possible.

Because only a limited amount was available from many of the samples, the official methods for iodine value and saponification equivalent were adapted to smaller sample size. A modification of the method of Sims and Stone (25) was used to determine iodine values with 5–8-mg. samples. Hanus reagent and starch indicator were substituted for Wijs solution and Thyodene indicator, respectively, and the concentration of sodium thiosulfate was increased to 0.1 *N*. Using a syringe pipette, 1.00 ml. of the volatile Hanus reagent was added rapidly to each sample dissolved in 1.0 ml. chloroform. The weighing bottles containing the samples were sealed with 15% potassium iodide solution and stored in the dark for 30 min., meanwhile being swirled at 5-min. intervals. Then, 0.5 ml. of 15% potassium iodide and 2.0 ml. of water were added to each. While the contents were magnetically stirred by a ¾-in. piece of stainless steel wire, the samples were titrated with a micro-burette. The pipette and burette were modifications of those described by Neish (20). The mean of 24 replicate iodine values (each with its own blank) was 31.98 (macro 32.00), with a standard deviation of 0.362. The accuracy compares favorably with that reported for the Sims and Stone method.

Saponification equivalents, which correspond to one-third the average molecular weight of the triglycerides in a fat, were calculated as follows:

$$\text{Saponification equivalent} = \frac{56.104}{\text{saponification number}} \times 1{,}000.$$

Saponification numbers were obtained by the semimicro procedure of Sims and Stone, except that the above micro-burette was used for the titrations. The mean of 33 replicate determinations (each with its own blank) was 232.5 (macro 233.1), with a standard deviation of 1.464. Coefficients of variation for both the iodine value and saponification number methods were satisfactorily low (0.113 and 0.629%, respectively).

The methanolysis procedure previously used to prepare methyl esters of fatty acids (26) was adapted to the semimicro scale. Two-thirds of a volume of methanol, containing 1.15 mg. potassium hydroxide per milliliter, was added to one volume of 20% fat in pentane. The mixture was refluxed for 1 hr., and then stored in the dark for 24 hr. The pentane solution containing the dissolved methyl esters was washed with successive lots of water and then dried over anhydrous sodium sulfate. Most of the pentane was removed by evaporation in a stream of nitrogen before the samples were examined by gas-liquid partition chromatography. There was no difference in the mole percentages of methyl esters prepared from the same milk fat by the semimicro method of methanolysis compared to the macro method.

Gas-liquid partition chromatography of the fatty acid methyl esters was carried out with an instrument [5] equipped with a 30-in. stainless steel column

[5] Aerograph Model A-100, Wilkins Instrument and Research, Inc., P. O. Box 313, Walnut Creek, Calif.

(¼-in. O.D.) containing 30% silicone as fixed phase on 70% fire brick (30–60 mesh). The operating temperature was 232° C. and the flow rate of helium was approximately 100 ml. per minute. After the sample (containing 8–20 mg. methyl esters) was injected, the chart drive was started at the initial response-to-air peak, which occurred about 15 sec. later. The separation of the methyl esters was recorded with a 10-mv. full-scale sensitivity recorder, filament current 200 ma., and chart speed 16 in. per hour. The retention time corresponding to each ester in the homologous series from methyl caprate to methyl stearate was determined under the above conditions and these values were used to identify the component esters in the samples. To express the methyl ester concentrations in mole per cent, the areas under the peaks were integrated by multiplying peak height times half-band width.

The micro method used to determine polyunsaturated fatty acids was essentially that of Herb and Riemenschneider (16). Aliquots of pentane containing known amounts of lipid (approximately 10.00 mg.) were evaporated and the residue dissolved in purified iso-octane. Conjugated acids were determined in the usual way. In the determination of the nonconjugated constituents, the aliquots (containing approximately 10.00 mg. lipid) were evaporated in the micro reaction tubes before 5 g. of 21% potassium hydroxide-glycol reagent at 25° C. was added. The time of heating at 180° C. was increased from 15 to 25 min. to allow for the heating-up period. The absorptivity present before isomerization was subtracted from the absorptivity after isomerization at 233, 268, 315, and 346 mμ. Nonconjugated acids were calculated from the equations under "*(c)*" of Herb and Riemenschneider's micro method. Values obtained by this procedure checked with results obtained by Herb and Riemenschneider's corresponding macro technique (15). The approximate amounts of monoethenoid acids were calculated as per cent of "oleic" acid from the difference between the iodine value of each sample and the unsaturation accounted for by the polyunsaturated constituents (5).

Solvent systems. Dutton has reviewed the characteristics desirable for solvent systems for countercurrent distribution. Unfortunately, any choice of solvents is largely empirical, and there is no substitute for experimentation. After considerable study of solvent systems that might prove useful, three systems were chosen for further investigation in the countercurrent distribution of milk fat. They were: a 22:7:3:2 (by volume) mixture of pentane, 2-nitropropane, nitromethane, and nitroethane; a 3:1:1 mixture of petroleum ether, furfural, and 2-nitropropane, and a 12:7:5 mixture of pentane, 2-nitropropane, and nitromethane.

Pentane was purified by percolation through a column of silica gel (28), and the nitroparaffins [6] were filtered before use. Furfural [7] was distilled under reduced pressure, and 0.01% hydroquinone added to retard oxidation. Each solvent system was prepared in a 25-liter flask, shaken to attain equilibrium,

[6] Commercial Solvents Corp., San Francisco, Calif.

[7] Quaker Oats Co., Chicago, Illinois.

and allowed to separate into two phases. The distribution coefficient of milk fat in each system was, respectively, 1.8, 1.2, and 3.0.

RESULTS AND DISCUSSION

Countercurrent fractionation of milk fat. In the first distribution shown (Figure 1), the solvent system was pentane, 2-nitropropane, nitromethane, and nitroethane. Five liters of upper phase was placed in the central reservoir, and 40 ml. of lower phase in each of the 100 cells of the apparatus except Cell 0, the first tube in the train. Then 4.0 g. of milk fat (iodine value 32.32) dissolved in 35 ml. of upper phase was introduced into a graduated separatory funnel. Lower-phase solvent was added with shaking until the volume of the lower phase was 40 ml., and then the contents of the separatory funnel were placed in Cell 0. The entire assembly was tilted back and forth on its horizontal axis 12 times, to equilibrate the lipids between the two phases. The phases were allowed to separate for 13 min., and the upper phase was decanted into the upper decantation cell by tilting the assembly upward. Then, by tilting the assembly downward again, the upper phase was transferred to Tube 1, its neighbor on the right. During this process, 40 ml. of fresh upper phase was added to Cell 0 by means of the filling device. The first three or four transfers were performed by hand, making any necessary adjustment of interface level by removing small amounts of lower phase just ahead of the lead tube (11). Then, the automatic driving mechanism was employed to repeat the entire process a total of 100 times. Finally, the contents of each tube were removed

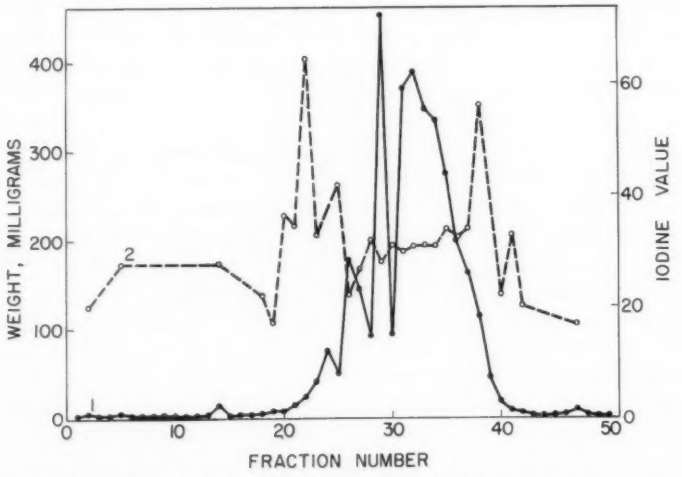


FIG. 1. Countercurrent distribution of milk fat with a 22:7:3:2 mixture of pentane, 2-nitropropane, nitromethane, and nitroethane—4.0-g. sample, 100 transfers at 15° C.

(1) Weight distribution curve.

(2) Iodine value curve.

with a 50-ml. hypodermic syringe, and consecutive pairs of samples were combined to obtain 50 fractions for analysis. This procedure is known as the "fundamental" method of operation (7).

The weight distribution curve in Figure 1 has three partially separated major peaks, which represent groups of glycerides with slightly different distribution coefficients. Iodine values of the fractions analyzed ranged from 17.17 to 64.61 and indicated that comparatively unsaturated small fractions preceded and followed the group of major peaks. Unfortunately, this promising solvent system was unsatisfactory at temperatures above 15° C., because of phase instability. Since the temperature of the room containing the countercurrent distribution apparatus could not be kept below 15° C., this system was not studied further.

With the solvent system of petroleum ether, furfural, and 2-nitropropane, used in a similar fundamental distribution, less peak separation was achieved, and it was difficult to remove the last traces of furfural from the fractions. Therefore, the use of this solvent system was discontinued also.

With the solvent system of pentane, 2-nitropropane, and nitromethane the fundamental distribution (Figure 2) shows two partially separated major peaks. Iodine values ranged from 8.30 to 45.25, and the most highly unsaturated fractions again preceded and followed the main peaks. This system was adopted for subsequent work. It was stable at temperatures between 15 and 20° C., and the phases separated more rapidly than with the first solvent system tested, though less peak separation was obtained.

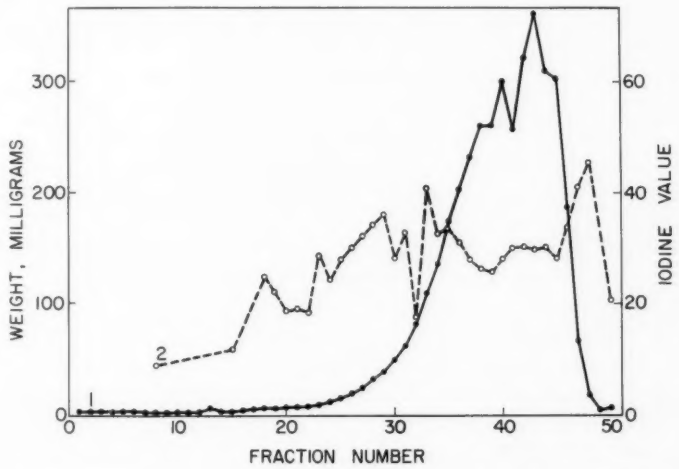
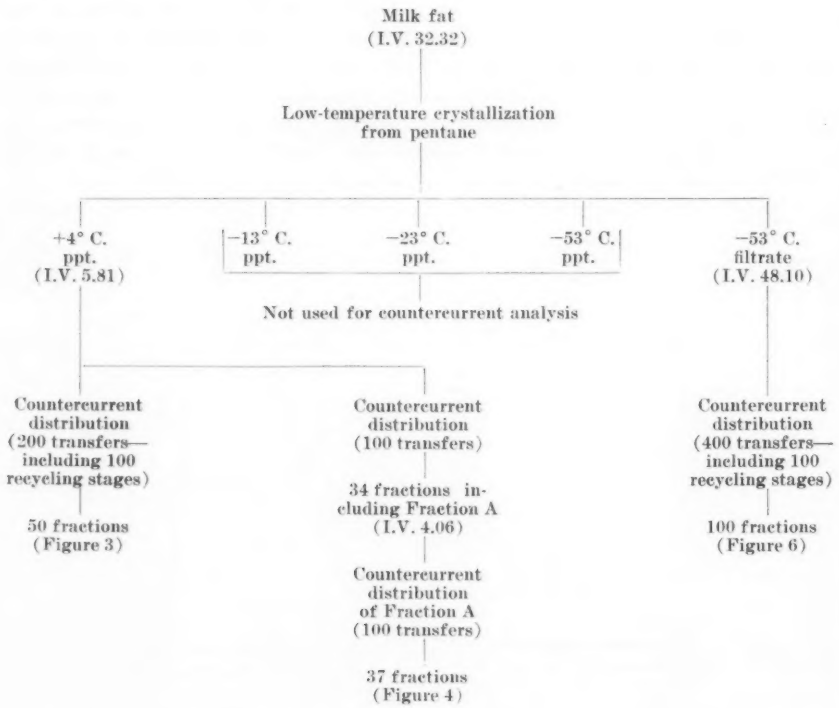


FIG. 2. Countercurrent distribution of milk fat with a 12:7:5 mixture of pentane, 2-nitropropane, and nitromethane—4.0-g. samples, 100 transfers at 20° C.

(1) Weight distribution curve.

(2) Iodine value curve.

The application of 100 transfers in the above countercurrent distributions of milk fat yielded triglyceride fractions with both lesser and greater saturation than the original fat. The range of saturation of these samples was comparable to that achieved by fractional crystallization of milk fat from pentane at low temperature (14), but only small fractions could be prepared conveniently. Figures 1 and 2 indicate that a much larger number of transfers would be necessary to separate the fat more completely into groups of triglycerides with close partition coefficients. However, the number of transfers that can be applied conveniently is limited by the number of cells available and the amount of solute in each cell required for analysis at the end of the distribution. Therefore, it was decided to employ a preliminary separation by fractional crystallization to obtain fractions less complex than the original fat, in amounts large enough to allow subsequent application of countercurrent distribution techniques. The scheme below shows the separation and the samples subjected to countercurrent distribution.



Countercurrent fractionation of +4° C. precipitate. This fraction (iodine value 5.81) was chosen for countercurrent distribution because it contained a high proportion of the relatively saturated triglycerides of milk fat. After

completing 100 transfers in the usual way with the solvent system adopted, the last transfer tube of the train was connected with Tube 0, so that the upper phases would enter this tube and be recycled instead of being ejected from the apparatus. One hundred recycling stages were applied, and then the 100 upper phases were removed with a 50-ml. syringe. Consecutive pairs were combined to obtain 50 fractions. The weight distribution curve is shown (Figure 3). Only one peak was separated, but the right-leg fractions of the curve were more saturated than the left-leg fractions. Furthermore, the saponification equivalents were higher for Fractions 42 and 44 (278.8, 285.3) than for Fractions 32 and 34 (252.7, 255.1). This shows that the more saturated glycerides, with higher molecular weights, migrated the fastest. The peak probably consisted of a large number of triglycerides with very close partition coefficients. About 15% of the sample remained in the lower phase.

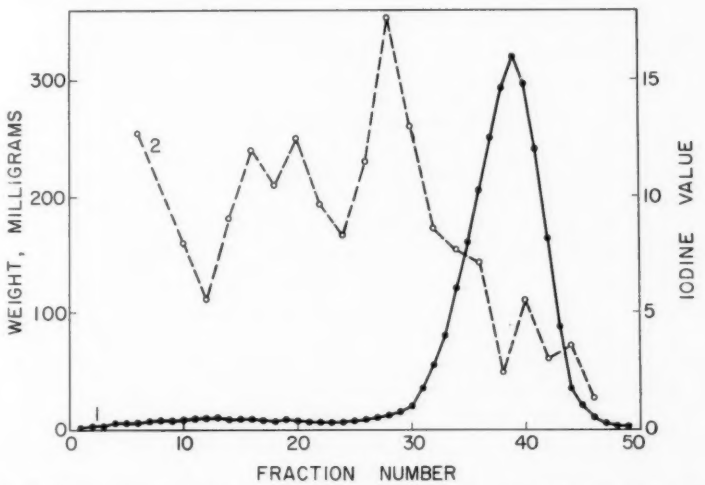


FIG. 3. Countercurrent distribution of +4° C. precipitate obtained by crystallization of milk fat from pentane, with a 12:7:5 mixture of pentane, 2-nitropropane, and nitromethane—3.0-g. sample, 200 transfers at 19° C.

(1) Weight distribution curve.

(2) Iodine value curve.

In another experiment, a highly saturated fraction, obtained by countercurrent distribution of the +4° C. precipitate, was fractionated further, in a second distribution. After completion of 100 transfers with 4.0 g. of the +4° C. precipitate, the first four phases flowing from the machine were combined and evaporated, giving a 1,300-mg. sample (Fraction A) with an iodine value of 4.06. Fraction A was again distributed in the system, and after 100 transfers the upper phases were removed from the cells. Consecutive groups of four phases were combined from Tubes 0 to 51, and consecutive pairs from Tubes 51 to 99, giving a total of 37 fractions.

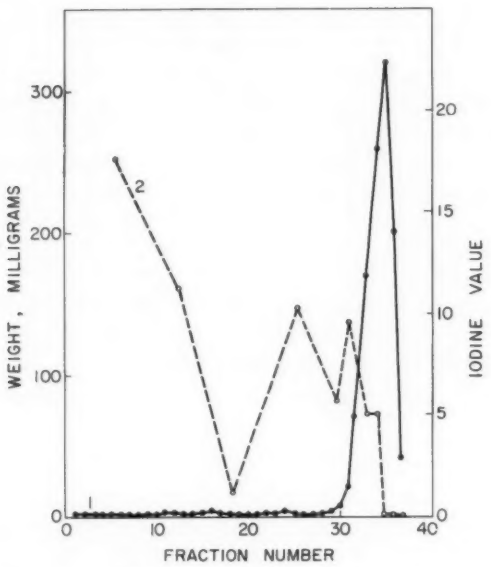


FIG. 4. Countercurrent distribution of Fraction A, obtained by countercurrent distribution of +4° C. precipitate, with a 12:7:5 mixture of pentane, 2-nitropropane, and nitromethane—1.2-g. sample, 100 transfers at 18° C.

(1) Weight distribution curve.

(2) Iodine value curve.

The weight distribution curve (Figure 4) shows separation of only one peak, but the glycerides that migrated the fastest (Fractions 37, 36, 35) were practically fully saturated (iodine values 0.04, 0.10, 0.11). Small amounts of relatively unsaturated glycerides trailed the peak. About 9% of Fraction A remained in the lower phase. It is probable that this material and the corresponding unrecovered solute in the previous experiment tended to remain in the lower phase because they were more polar than the glycerides recovered in the upper phase.

Analysis of most saturated fraction separated from +4° C. precipitate. The mixed methyl esters from a 20-mg. aliquot of Fraction 37 were separated by gas-liquid partition chromatography (Figure 5). Mole percentages of methyl laurate, myristate, palmitate, and stearate were 0.2, 3.6, 80.4, and 15.7, respectively. Only a trace of methyl caprate was detected and there was no methyl arachidate. The calculated saponification number of 204.5 for Fraction 37 checked satisfactorily with the experimental value of 205.3. This confirmed the absence of esters of lower molecular weight than methyl caprate. Since the iodine value was only 0.04, these data show that the triglycerides of Fraction 37 were composed essentially of only four saturated fatty acids—lauric, myristic, palmitic, and stearic.

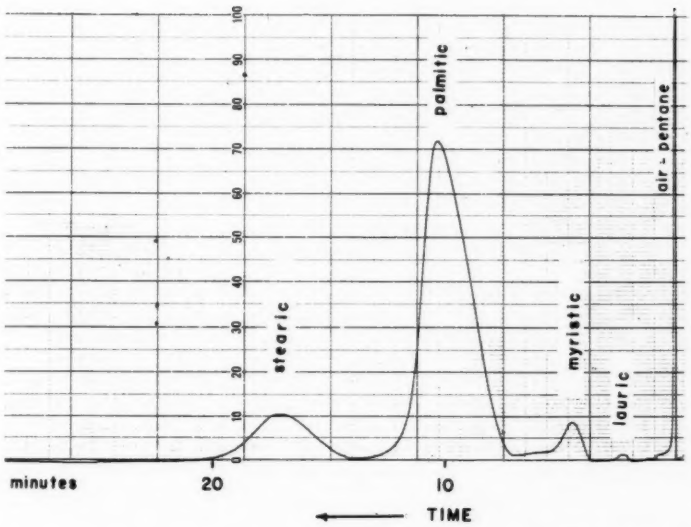


FIG. 5. Gas-liquid partition chromatogram of fatty acid methyl esters from the most saturated fraction obtained by countercurrent distribution of +4° C. precipitate.

The possible component triglycerides of Fraction 37, by any calculation (19, 27), must include tripalmitin, because there is more than 66.67 mole per cent of palmitic acid in the fatty acid mixture. Appreciable amounts of dipalmitostearin and dipalmitomyristin also must be present in this fraction. These data provide strong evidence for the presence of the simple triglyceride tripalmitin in milk fat, in addition to dipalmitostearin and dipalmitomyristin.

Countercurrent fractionation of −53° C. filtrate. This material (iodine value 48.10) was chosen for countercurrent fractionation because it contained a high proportion of the relatively unsaturated triglycerides of milk fat. To obtain fractions large enough for chemical analysis, the same distribution was performed twice, with 4.0 g. of sample each time. After the initial 100 transfers, 100 recycling stages were applied. Then sample collection was begun, while new upper phase was metered into Cell 0 during each of the next 100 transfers. When 200 upper phases had emerged from the machine, consecutive pairs were combined, giving a total of 100 fractions. In this procedure, the constituents that migrate fastest are found in those upper phases first discharged from the machine. The above 400 transfers eluted practically all the solute from the lower phases. The 100 fractions from the second run were combined with the corresponding fractions of the first distribution.

The weight distribution curve (Figure 6) shows partial separation of three major peaks with the more highly unsaturated glycerides migrating slowest. No attempt was made to follow the distribution of the unsaponifiable constituents, but it was observed that the carotenoids tended to remain in the lower

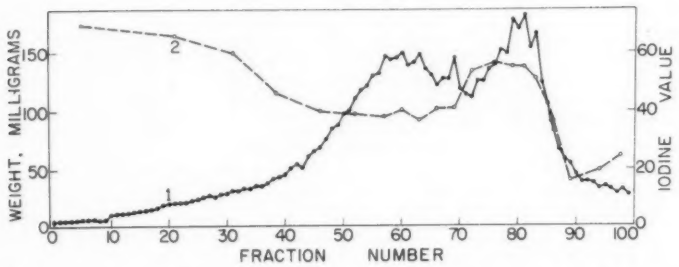


FIG. 6. Countercurrent distribution of −53° C. filtrate obtained by crystallization of milk fat from pentane, with a 12: 7: 5 mixture of pentane, 2-nitropropane, and nitromethane—8.0-g. sample, 400 transfers at 19° C.

(1) Weight distribution curve.

(2) Iodine value curve.

phase. Figure 6 resembles the weight curves obtained by countercurrent distribution of linseed oil (11) and soybean oil (23), except that less resolution of peaks was obtained. Although greater numbers of transfers were employed in the distribution of these oils than with the −53° C. filtrate from milk fat, they had not been subjected to preliminary fractionation. The −53° C. filtrate probably contained a greater number of different fatty acids (17) and a greater variety of triglycerides than these vegetable oils. Therefore, a comparatively larger number of transfers would be necessary to resolve a greater number of smaller peaks in the −53° C. filtrate.

TABLE 1

Analyses of representative fractions from countercurrent fractionation of −53° C. filtrate

Fraction	Saponification equivalent	Fatty acids						
		Conjugated diene	Nonconjugated				Total polyethenoid	Total monoethenoid[a]
			Diene	Triene	Tetraene	Pentaene		
		(weight %)						
0–9	288.3	0.4	1.3	0.6	0.0	0.2	2.5	70.7
20–22	252.7	0.8	2.1	1.9	0.4	0.3	5.5	58.3
30–32	231.6	1.1	5.0	4.3	1.1	0.8	12.3	32.9
38–39	217.4	1.3	5.4	4.2	1.2	0.8	12.8	16.8
46	229.4	0.7	3.5	1.6	0.5	0.3	6.6	27.7
52	224.3	0.3	3.4	2.0	0.6	0.7	7.0	24.6
57	247.2	0.2	2.1	0.8	0.3	0.2	3.4	33.3
63	241.3	0.3	1.8	1.0	0.3	0.3	3.6	32.1
69	252.2	0.5	1.9	2.1	0.6	0.3	5.4	31.0
76	274.3	0.8	4.6	1.6	0.6	0.3	7.9	43.3
81	272.4	0.7	3.4	0.8	0.3	0.1	5.4	49.2
86	242.6	1.5	4.4	0.8	0.3	0.2	7.2	23.7
89	210.8	....	....	....	....	....	....	....
93–94	197.2	1.6	5.5	1.5	0.4	0.3	9.3	0.0
97–99	191.0	1.3	6.9	2.3	0.4	0.2	11.1	2.8

[a] Calculated as per cent oleic acid from the difference between the iodine value and the unsaturation accounted for by the polyunsaturated constituents.

Distribution of fatty acids in −53° C. filtrate. Table 1 lists saponification equivalents, individual polyethenoid fatty acids, and total polyethenoid and monoethenoid acids of representative fractions from the above countercurrent distribution. The amounts of conjugated triene and tetraene acids were quantitatively negligible and are not shown. The amounts of monoethenoid acids ranged from 70.7 to 0.0% calculated as oleic acid, although some C_{10}–C_{16} monoethenoid acids undoubtedly were also present.

The saponification equivalents representative of Fractions 0–22 were higher (288.3–252.7) than those (210.8–191.0) representative of Fractions 89–99. In Fractions 0–22, most of the unsaturation was due to monoethenoid acids and not to polyethenoid acids, whereas the reverse was true for Fractions 86–99. These data show that the monoethenoid acids were associated with the higher molecular weight saturated acids in Fractions 0–22, whereas the polyethenoid acids were associated with the lower molecular weight saturated acids in Fractions 89–99. Most of the intermediate fractions follow corresponding trends. These results indicate that there is some degree of selectivity of fatty acids during the formation of milk fat triglycerides.

Analysis of most unsaturated fraction separated from −53° C. filtrate. The mixed methyl esters from a 9-mg. aliquot of combined Fractions 0–9 were examined by gas-liquid partition chromatography (Figure 7). Mole percentages of the C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} esters were 0.9, 2.5, 11.0, 43.0, and 42.6, respectively.

Since the conclusion seems justified that tripalmitin is present in milk fat, one may use the above data to speculate also on the presence of triolein. The

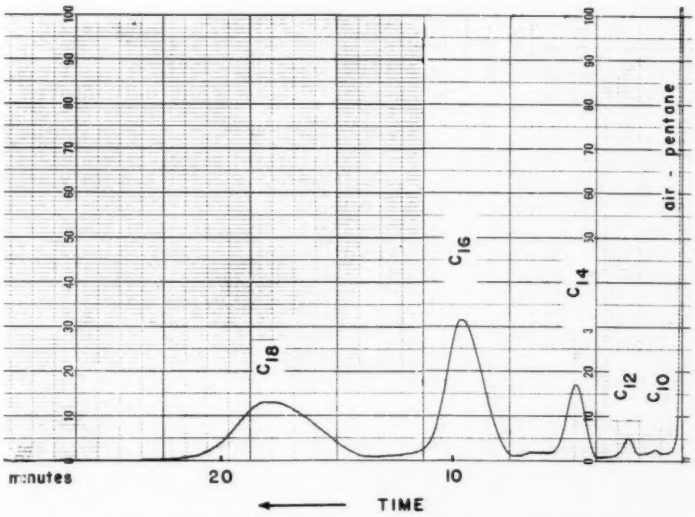


FIG. 7. Gas-liquid partition chromatogram of fatty acid methyl esters from the most unsaturated fraction obtained by countercurrent distribution of −53° C. filtrate.

spectrophotometric analyses (Table 1) for Fractions 0–9 show that the C_{18} peak included approximately 2% of C_{18} polyethenoid constituents. Furthermore, with the column (30% silicone as partition medium) and conditions (232° C., helium flow rate 100 ml. per minute) employed in the chromatographic separation, the individual C_{16}–C_{18} peaks may have included both monoethenoid and saturated components of the same chain length. Unfortunately, there was insufficient material available to collect samples corresponding to the individual peaks for further examination by chemical or spectrophotometric methods. The peak of interest here is the C_{18} peak. It is highly improbable that it would contain a higher ratio of stearic to oleic methyl esters than the 1:4.4 ratio Jack and Henderson (17) found in their study of a −53° C. filtrate from milk fat. Even if the C_{18} peak contained this same ratio, then no more than 9.2 mole per cent ($1/4.4 \times 42.6$) would be stearic ester. Thus, 42.6 mole per cent − (2.5 mole per cent polyethenoid + 9.2 mole per cent stearic) = 30.9 mole per cent oleic ester. Since, in a distribution pattern whereby the fatty acids are distributed as widely as possible (even), an excess of a single acid over 66.67 mole per cent is necessary to assure the presence of a simple triglyceride, and there is present here only approximately 31 mole per cent of oleic acid, if this pattern prevails, then triolein can not exist. However, since the data indicate that there is some degree of selectivity in the arrangement of *(a)* the monoethenoid and the higher molecular weight saturated acids and *(b)* the polyethenoid and the lower molecular weight acids, the strict observance of this pattern must be ruled out. If oleic acid follows the same glyceride distribution as palmitic, which it approximates in amount in milk fat, the occurrence of triolein can not be excluded.

Additional work is in progress to determine more completely the composition of milk fat glycerides, using the experience gained here and taking advantage of gas-liquid chromatography techniques (22) reported since the completion of this work.

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RADIATION PRESERVATION OF MILK AND MILK PRODUCTS. V. PRECURSORS TO THE RADIATION-INDUCED OXIDATION FLAVOR OF MILK FAT[1]

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SUMMARY

Oxidation flavor produced upon irradiation of milk fat is derived from certain of its component fatty acids. One of these acids has been identified as linolenic acid. Other acids required for production of irradiation-induced oxidation flavor have been isolated in an unidentified mixture. This unknown isolate is believed to contain the vinyl group and branched chains.

Linolenic acid produced a fishy flavor upon irradiation, while the mixture of fatty acids containing the vinyl group and branched chains gave rise to a flavor characterized as candle-like. These two flavors blended upon mixing to give the typical tallowy oxidation flavor of irradiated milk fat.

In the course of investigations (9, 4, 16, 17) carried out in this laboratory on the effects of radiation on milk and milk products, it has been shown (18) that irradiation-induced oxidation flavor of butterfat is most strongly produced in the unsaturated glycerides. Therefore, it seemed reasonable to expect that a study of the component fatty acids of these glycerides should yield information concerning the identity of the compounds which on irradiation produce oxidation flavor. It also seemed reasonable to suppose that a similar study of milk fatty acids should lead toward identification of precursors of the disagreeable oxidation flavor developed in milk upon its irradiation. Results of such an investigation of the unsaturated fatty acids of milk fat are reported in this paper.

EXPERIMENTAL PROCEDURE

Preparation of milk fat. Forty per cent heavy cream, purchased locally, was churned at 15° C. and the fat allowed to separate at 45° C. The liquid fat was drained off, washed twice with water, and filtered through anhydrous sodium sulfate. The yield of milk fat was greater than 90%.

Saponification of milk fat and isolation of fatty acids. Anhydrous milk fat (600 g.) in 1,500 ml. of a 12% solution of potassium hydroxide in absolute ethanol, was heated under reflux in an atmosphere of nitrogen for 1 hr. Alcohol was removed by distillation at aspirator pressure. Unsaponifiable material was not removed because of emulsion formation. Acids were liberated from the soaps

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by the addition of dilute sulfuric acid and washed with warm water until free of mineral acid. Lower fatty acids were removed by steam distillation at 50° C. and 10–15 mm. Hg for 5 hr. The residue thus obtained contained unsaponifiable material and nonvolatile fatty acids. Its odor indicated that traces of lower fatty acids were present.

Fractionation of the fatty acid mixture. The fractionation scheme is shown in Figure 1. Operations were carried out under nitrogen whenever possible.

Removal of saturated fatty acids by crystallization at 0° C. (6). The fatty acid mixture (500 g.) in 1,500 ml. of methanol was allowed to stand in an ice bath at 0° C. overnight. The solid product, presumably saturated fatty acids, was obtained in 40% yield. The filtrate, which contained 60% of the original fatty acid mixture, was brought to a total volume of 2 liters with methanol.

Fractionation of unsaturated fatty acid mixture by urea adduct formation (12). The methanol solution was treated with 260 g. of urea and heated to boiling. The resulting solution was allowed to come to room temperature. The urea adduct thus obtained was removed by filtration and the filtrate again treated with urea (160 g.), as just described. A total of five crystalline urea adducts was obtained in this manner. Additional quantities of urea used were 160, 200, and 200 g., respectively. The adducts thus obtained were designated A, B, C, D, and E, while the final methanol solution was called F.

Regeneration of fatty acids from Fractions A through E was accomplished by dissolving the adducts in hot dilute acid and extracting with pentane. The pentane solutions were dried over anhydrous sulfate and evaporated under reduced pressure. The fatty acids so obtained were stored under nitrogen at −29° C. until their subsequent irradiation and flavor evaluation.

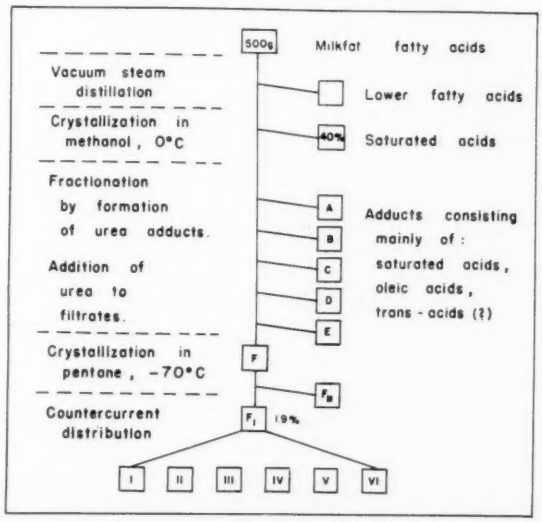

FIG. 1. Fatty acid fractionation scheme.

Fatty acids in F were obtained by evaporation of methanol under reduced pressure, removal of urea in hot dilute acid, and extraction of the acids with pentane. The pentane solution was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue, F, contained 15 g. and represented a 3% yield from the milk fatty acid mixture.

Irradiation and flavor evaluation of Fractions A-F. The fraction (usually 10 mg.) to be evaluated was dissolved in 1 ml. of mineral oil (Nujol) and irradiated with 2×10^6 rep (Co^{60} source). The irradiated material was diluted to 100 ml. with homogenized whole milk and mixed in a Waring Blendor for 2 min. Flavor evaluation was carried out on the emulsion thus obtained or, if intensity was too great, on emulsions obtained after further dilution with milk.

A panel of five judges was presented with the samples thus prepared, together with a control milk sample, and asked to indicate the approximate intensity and types of flavor of each of the samples. Fractions D and F were found to exhibit the oxidation flavor, with F possessing the greatest intensity of flavor. The flavor of E was typical of that associated with lower fatty acids.

Fractionation of F by low-temperature crystallization. Fraction F (9.6 g.) was dissolved in 100 ml. of pentane and held at −70° C. for 4 hr. The resulting solid, 3 g. (Fraction FII), was removed by filtration. The filtrate was evaporated under reduced pressure and the residue (Fraction FI) isolated (6.2 g.). The yield of FI from the milk fatty acid mixture is 1.9%.

Examination of Fractions F, FI, and FII by the method of Brice and Swain (5) showed that FI contained the highest proportion of unsaturated fatty acids (Table 1).

Fractionation of FI by counter-current distribution. The procedure of Ahrens and Craig (2) for the quantitative separation of oleic, linoleic, and linolenic acids was followed with certain modifications described below. Distribution was carried out in a 200-tube automatic apparatus with the solvent system: heptane, methanol, formamide, acetic acid (3:1:1:1).

Fraction FI (2.1 g.) was dissolved in sufficient volume of the upper solvent phase to fill 20 tubes. After 180 transfers had been carried out, the distribution pattern was obtained by determination of the weight of material present in a

TABLE 1

Determination of unsaturation in Fractions F, FI, and FII

	F	FI	FII
	(%)		
Diene			
Conjugated	3.82	4.84	2.24
Nonconjugated	13.35	18.02	3.54
Triene			
Conjugated	0.120	0.137	0.075
Nonconjugated	18.56	27.00	2.38
Tetraene			
Conjugated	0.020	0.025	0.018
Nonconjugated	9.73	13.70	1.62
Total polyunsaturation	45.60	63.72	9.87

1-ml. aliquot withdrawn from the upper phase in each tube. Each aliquot was placed in a small aluminum cup patterned after the glass shells of Craig *et al.* (7), and evaporated in a stream of nitrogen by heating for 3 min. with an infrared lamp at a fixed distance. The cups were allowed to cool for 3 min. before weighing and were stored on special cardboard racks in a desiccator.

Four major peaks having partition ratios of 0.51, 1.50, 2.60, and 9.60, respectively, were obtained. Based on the shape of the curve, none of these substances could be considered pure. The material having a partition ratio of 9.60 was believed to be an unsaponifiable fraction because of its color and solubility. It was consequently removed from the apparatus. The remaining substances were recycled until 298 transfers had been accomplished. A distribution pattern containing six peaks was obtained (Figure 2). Separation was not complete, as shown by the overlapping of these peaks.

In order to obtain the purest component of these fractions, the upper phase of the ten central tubes in each peak was removed and evaporated under reduced pressure. The residue was dissolved in petroleum ether, washed with water to remove acetic acid, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residues were weighed and then stored under nitrogen at −29° C. The weights obtained in the ten central tubes of Fractions I, II, V, and VI were 0.264, 0.064, 0.055, and 0.156 g., respectively. Partition ratios of Fractions I through VI were 2.55, 4.45, 8.04, 0.49, 0.94, and 1.52, respectively.

Infrared spectra (Figure 3) were determined in 10% carbon tetrachloride solution by means of a Perkin-Elmer Model 21 double-beam spectrophotometer fitted with rock-salt optics.

The spectrum of a pure sample of linolenic acid [iodine value (Wijs); 273.0: theoretical value; 273.51: conjugated polyunsaturated constituents ex-

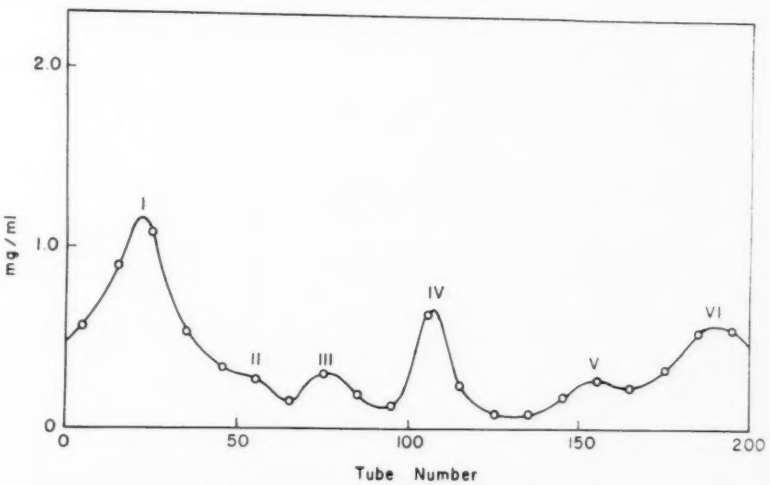

FIG. 2. Distribution pattern of Fraction FI.

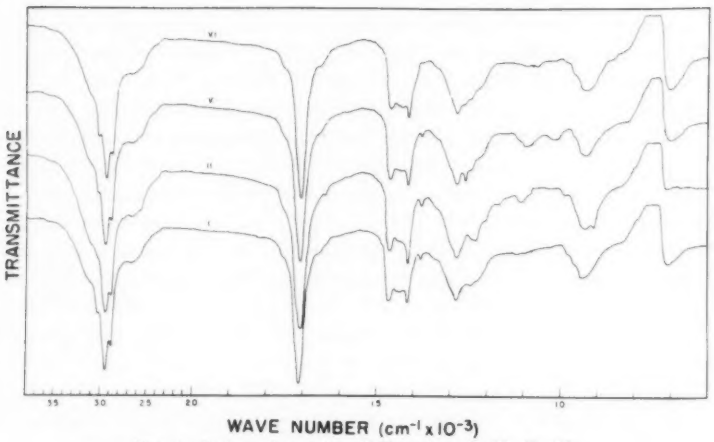

FIG. 3. Infrared spectra of Fractions I, II, V, VI.

pressed as percentage of C_{18} fatty acids; dienoic, not more than 0.15% trienoic: nil: tetraenoic: nil)], was determined in the same manner.

Irradiation and flavor evaluation of Fractions I through VI were carried out in the manner previously described. Results of the flavor evaluation are shown in Table 2.

RESULTS AND DISCUSSION

In view of the evidence (18) that irradiation-induced oxidation flavor of butterfat is produced most strongly in the unsaturated glycerides, it appeared that information concerning the identity of the component unsaturated fatty acids would bring nearer eventual solution of the many problems related to irradiation of fats. It also appeared by analogy that a similar study of the unsaturated acids of milk fat should lead to useful information concerning the identity of precursors to the irradiation-induced oxidation flavor of milk. The investigations described in this paper have been carried out with this goal in mind.

As stated previously, the pattern (Figure 2) obtained from counter-current distribution of Fraction FI indicated that none of the six peaks represented a pure compound.

TABLE 2

Flavor evaluation of irradiated fatty acid fractions and esters of pure acids

Fraction No.	Flavor of milk terms used by judges
I	Slightly off
II	Slightly soapy, candle-like
III	No off-flavor
IV	Soap, sharp
V	Fishy, cod liver oil
VI	Strong fishy, cod liver oil
Methyl oleate	Waxy
Methyl linoleate	Burnt, papery, cardboard, nutty
Methyl linolenate	Fishy, cod liver oil, oily

Reference to the work of Ahrens and Craig supported this view, since 650 transfers had been required to separate a mixture of oleic, linoleic, and linolenic acids. It would seem logical that at least that number of transfers would be required to separate the more complex mixture found in FI.

In spite of possible impurity of the six components obtained by countercurrent distribution, the contents of the upper solvent phase in the central tubes of each peak were isolated. Irradiation failed to produce oxidation flavor in any of the six isolates (Table 2). A mixture of all fractions, however, produced a flavor similar to oxidation flavor. By a process of elimination it was found that a mixture of Fractions II and VI in proportions of 1 to 3 or 2 to 3 exhibited an oxidation flavor which could not be distinguished from that produced by a sample of irradiated milk fat emulsified in milk. Therefore, it was concluded that the components present in II and VI were precursors to the irradiation-induced oxidation flavor of milk fat.

Fractions III and IV were believed, on the basis of general properties, to contain unsaponifiable material and lower fatty acids, respectively. These fractions were not examined further.

Since the quantity of Fractions I, II, V, and VI isolated was small, infrared spectra provided the major evidence of their chemical identity. It was hoped that neutral equivalents, iodine values, proportions of polyunsaturation, and elemental analyses could be obtained. These determinations would yield information concerning the chain length and homogeneity of the fractions. However, such determinations must wait until more material can be prepared.

Interpretation of infrared spectra. The infrared spectra (Figure 3) of I, II, V, and VI are typical (1, 3, 15) of solution spectra of unsaturated fatty acids. Little more than this can be concluded about the identity of Fractions I and V, other than that they are not *trans*-acids, since there is no band at 965-975 cm^{-1} in their spectra. Information concerning the molecular weight and percentage of unsaturation would aid greatly in prediction of these structures.

The spectra of Fractions II and VI, however, are more informative. VI has been identified as linolenic acid having the double bonds in the *cis*-configuration. Identification was made by comparison of the spectrum of VI with that of an authentic sample[2] of linolenic acid of known purity. It is, therefore, concluded that linolenic acid is a precursor to irradiation-induced oxidation flavor.

Linolenic acid has previously been reported (11, 13) to be present in butterfat in amounts ranging from 1 to 3%.

The spectrum of Fraction II indicates that the vinyl group as well as branched chains are present. Since the homogeneity of II has not been established, it can not be assumed that a single branched-chain, vinyl acid is present. Evidence (3) for the vinyl group are the bands at 1,640 cm^{-1}, 1,415 cm^{-1}, 995-985 cm^{-1}, and 915-910 cm^{-1}. Evidence for chain-branching is provided by bands at 1,285 cm^{-1} (8), 1,235 cm^{-1} (8), 1,170 cm^{-1} (3), and 1,380 cm^{-1} (3). At the present time only one vinyl acid, caproleic acid (9, 10-decenoic acid), is known

[2] This sample was obtained from the Hormel Foundation, Austin, Minnesota.

(10) to occur in milk fat. Further investigation of Fraction II is desirable to determine if caproleic or a heretofore unknown vinyl acid has been isolated. The presence of several branched-chain saturated acids in butterfat has been reported (14). This fact, in addition to the probability of their presence in Fraction F by nature of their known resistance to urea complex formation, supports their possible presence in Fraction II. Again, further investigation of Fraction II should provide important information in this regard.

The relationship of irradiation-induced oxidation flavor to linoleic acid (Fraction VI) and Fraction II. Linoleic acid has been shown to be a precursor to irradiation-induced oxidation flavor. Confirmatory organoleptic evidence of this is the fact that pure methyl linolenate after irradiation exhibited a fishy flavor and that when mixed with an irradiated sample of Fraction II typical oxidation flavor was noted. Similar behavior was noted for Fraction VI (linolenic acid). These observations are outlined schematically in Figure 4. A definite

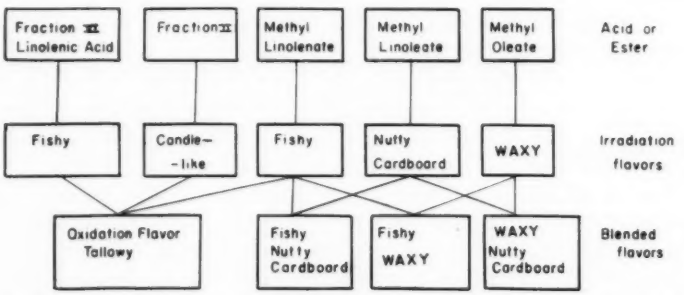

FIG. 4. Flavor of irradiation products of fatty acids and esters and their blended flavors.

change in flavor was observed when a mixture of the pure acid or ester was made with Fraction II. This blending effect or change in flavor was not noted when irradiated samples of pure methyl linolenate, methyl linoleate, and methyl oleate were mixed and evaluated (Figure 4). In these cases, the mixtures exhibited flavors in which the characteristic flavor of each component was recognized. It, therefore, appears that Fraction II possesses a unique blending effect.

Since no evidence exists that Fraction II is a single compound, it is assumed that it contains a mixture of acids characterized by the vinyl group and branched chains. No information is, thus, available as to whether one component or more than one component of Fraction II is a precursor to irradiation-induced oxidation flavor.

ACKNOWLEDGMENT

The authors wish to thank Dr. Arthur C. Cope of the Chemistry Department, M.I.T., for permission to use the counter-current distribution apparatus; Dr. Kenneth R. Henery-Logan of the same department for his advice on the operation of the apparatus, and Dr. Emily Wick, of the Department of Food Technology, M.I.T., for advice and assistance in the preparation of the paper.

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CIRCULATION CLEANING. II. EFFECTS OF ENTRAINED AIR

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SUMMARY

The effect of altering the density and viscosity of deterging mediums by antifoam additions was investigated. Such additions were essentially without effect on the pressure side of the system, and inhibited cleaning on the vacuum side. This latter is ascribed to a limiting of the scrubbing action of occluded air and bubbles. It was demonstrated that, under controlled conditions, cleaning is more effective on the vacuum side, and that the effectiveness is further increased by permitting air to leak into the system.

Interest in circulation cleaning, or cleaning-in-place, has been considerable in recent years. Of primary importance to the cleaning operation is turbulence, as shown in a previous publication (1). Since turbulence is a function of, among other things, the effective mean density and the effective mean viscosity of the circulating medium as it exists in this dynamic state, the formation of foam on the line would be expected to influence the cleaning operation. This might be overcome by adding an antifoam agent.

Such an inclusion would probably introduce two opposing influences through two changes in the circulating medium: 1) a higher effective mean density, giving greater turbulence for a given set of conditions; and 2) a higher effective viscosity, giving lower turbulence for a given set of conditions. These possibilities would hinge on the mode of action of the antifoam, i.e., whether its effect was inactivation of the surface-active agent in the detergent or competition for the solution-air interface. If the antifoam exhibited surface activity in its own right, it might also be attracted to the solution-metal interface, interfering with the cleaning operation. Experimental determinations with the Du Nouy tensiometer indicated the antifoam tested (Dow Corning AF) possessed some surface activity, and had essentially no effect on the surface activity of a detergent solution.

This work was directed toward studying the influence of foam formation and air inclusion in the circulating medium, and the effect of antifoam additives on the cleaning operation.

METHODS AND PROCEDURE

The methods previously described (1) were modified as follows:

Recirculating apparatus. Two-foot sections of 1½-in. glass pipe were attached to the ends of the pipe test section, and the unit arranged to permit operating this section on the pressure or vacuum side under variable flow conditions (Figure 1). The test section was operated in a horizontal position with the test disc at the top.

Milk films and cleaning evaluation. About 100 μc of a radioactive phosphorus solution was added to 100 ml. of homogenized milk. This preparation was further

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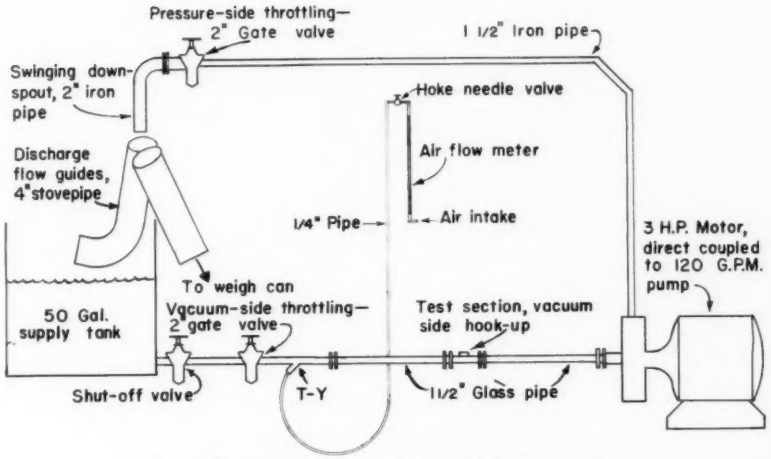

FIG. 1. Schematic diagram of recirculation apparatus.

diluted until milk films prepared from 0.5–1.0 ml. exhibited activities of 1,500–4,000 C.P.M.

The deterging medium was a 0.5% solution of a standard dairy cleaner, composed of 40% sodium metasilicate, 30% tetrasodium pyrophosphate, 20% sodium carbonate, and 10% Trend 40. The solution was maintained at 46° C. Runs were limited to 30.0 sec., so that enough soil was left for evaluation of variables.

Through-put was determined by diverting a portion from each run for a timed period through the swinging downspout to a weigh can. Thus, the flow could be diverted without changing the friction head of the system. The antifoam, used at 200 p.p.m., was thoroughly dispersed. After each trial, the surface of each test disc was carefully scoured with 0000 wool, and the residual activity (normally 50–70 C.P.M.) treated as background

RESULTS AND DISCUSSION

With the test section on the pressure side of the system (between the pump and the outlet), antifoam produced no detectable differences in cleaning (Figure 2, A-B). With and without antifoam, the solutions in the glass lines appeared the same (clear: no turbidity or foam).

When the test section was moved to the vacuum side of the system (between the inlet and the pump), considerable turbidity indicated large quantities of air incorporated into the system. This was minimized by placing a deflector on the discharge, thus preventing the pump from gulping this freshly aerated solution.

Figure 2, C-D, indicates that the antifoam interfered with cleaning on the vacuum side of the system. Because no such effect is apparent on the pressure side, this is probably not due to interference with the detergent or the solution-metal interface. If the antifoam functioned by orienting so as to minimize the

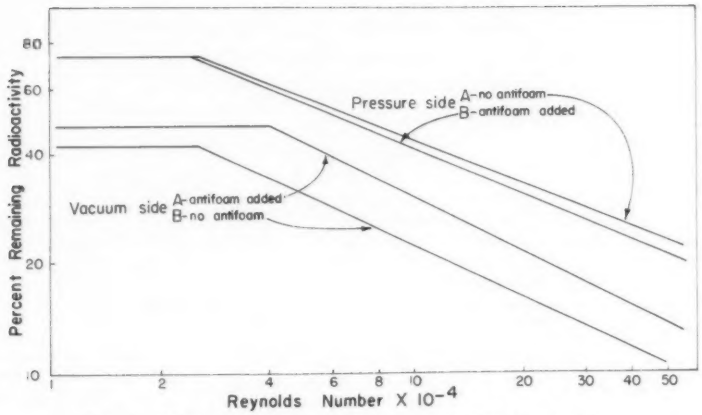

FIG. 2. Effect of antifoam additions on circulation cleaning.

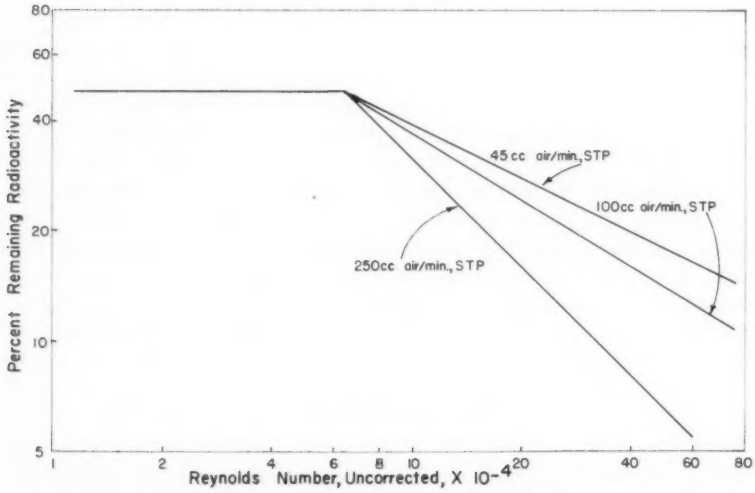

FIG. 3. Effect of air leaks on cleaning effectiveness, Reynolds numbers uncorrected.

solution-air interface, it would lessen the scrubbing action of bubbles and occluded air; this would affect the cleaning operation on the vacuum side only.

If this reasoning were correct, and occluded air contributed to cleaning effectiveness, then air leaks in the vacuum system might be expected to aid cleaning.

To check this point, detergent solutions with and without added antifoam were circulated with controlled and measured air leaks. Air flow was measured with a precision tube flow meter (Scientific Glass Apparatus Co., Model 150) and throttled with a Hoke needle valve.

When the deterging medium contained antifoam, air leaks had no noticeable

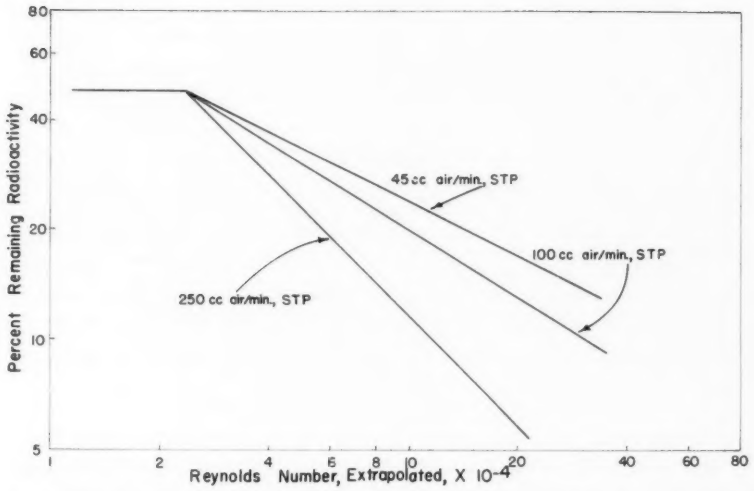

FIG. 4. Effect of airleaks on cleaning effectiveness, Reynolds numbers corrected by extrapolation.

effect on cleaning; in the absence of antifoam, the effect was striking. Although the effective density of the deterging medium could be calculated, it is virtually impossible to plot the data in terms of Reynolds numbers, for this would require measuring the viscosity of the circulating medium under these dynamic conditions. These data (no antifoam) are plotted with the abscissa as "Uncorrected Re" (Figure 3). It is interesting that the points were best satisfied by a system of straight lines intersecting at about Re 65,000. In all other cases, where viscosity and density of the circulating medium were known, in this and previous studies, intersection occurred at Re 25,000. This shift probably resulted from errors in the uncorrected Reynolds numbers. In Figure 4, these values have been corrected, shifting the intersection to its predictable position by multiplying by 25,000/65,000.

ACKNOWLEDGMENT

The technical assistance of R. C. Fowlks is gratefully acknowledged.

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ADSORPTION OF COPPER ON STAINLESS STEEL[1]

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SUMMARY

Under some conditions, dissolved copper becomes associated with stainless steel in a form that is readily available for contamination of milk. A small amount of copper-nickel alloy, either used or cleaned with stainless steel, can cause disproportionately large copper contamination of milk. Adsorption of dissolved copper on stainless steel was appreciable in the pH range of 6 to 10, especially in the presence of hypochlorite. The amount of copper that adsorbed increased with the concentration of copper in solution, the temperature, and the time of exposure. Copper adsorption was slight or absent at low or very high pH or in the presence of a chelating agent (EDTA). Adsorbed copper was not removed by thorough rinsing with water, but largely was removed by a chelating agent (EDTA) or milk, or exhaustively by hot nitric acid.

In both laboratory and commercial experiments, copper contamination has been observed in milk processed in equipment made entirely of stainless steel. A source of contamination was found to be copper-bearing alloys in other equipment cleaned in the same circulation cleaning circuit as the stainless steel. Part of the copper that dissolved from these alloys during cleaning became associated with stainless steel surfaces in a state readily removed by milk. This association of copper with stainless steel is referred to herein as adsorption, although its nature has not been established.

Swartling (10) described conditions in some butter factories wherein copper from the hot-water supply became deposited on equipment and later contaminated butter. Raw water took up considerable amounts of copper from heaters with copper pipes. Then, when the butter-manufacturing equipment was cleaned with hot water, copper deposited on the surface of the equipment, and later dissolved in cream and butter.

Hensley, Long, and Willard (5) reported on variables that influenced the adsorption of sodium ions on glass, and included some data on adsorption of sodium on fused silica, steel, platinum, silver, and aluminum. Although adsorption of metals on glass is well known in trace-element analysis (11), little has been reported on the adsorption of metal ions on metals.

This paper reports a study of certain variables that influence copper adsorption on, and desorption from, stainless steel surfaces. The data presented—only part of those obtained in this study—are similar to and consistent with results of other experiments that are not reported. Those presented were selected to illustrate the influence of some important factors.

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Literature has been reviewed pertinent to the relation of copper and its alloys to the development of oxidized flavor (1, 7, 8, 9). Copper-nickel alloys (white metals) have been used extensively in the dairy industry, even though they contaminate milk with copper and promote oxidized flavor (7, 13).

Experimental Procedure

Determination of copper. Copper determinations were made by a procedure that included dry-ashing of milk and colorimetric estimation of the copper as its carbamate (6).

Preliminary observations. The extent to which copper that is adsorbed on stainless steel may contaminate milk was illustrated by the following pilot-scale experiment: A steam-jacketed processing kettle was connected by sanitary fittings to a pump, with discharge from the pump returned to the kettle. All inside surfaces of the kettle and the circulation circuit were 18-8 type stainless steel, except that one stainless steel elbow in the cleaning circuit was replaced by a white metal (copper-nickel alloy) elbow for part of the experiment. An acid detergent[3] was circulated through the system for 5 min. at 140° F., the solution was drained, and the equipment was rinsed thoroughly. About 5 gal. of skimmilk was circulated in the equipment for 5 min. at 140° F., and removed to a stainless steel can. The copper concentration of the skimmilk was 0.080 p.p.m. Then, a white metal elbow was installed in place of a stainless steel elbow, and the same skimmilk was circulated again. The copper content increased to 0.107 p.p.m., an increase of 0.027 p.p.m. The skimmilk was again removed and the equipment was cleaned and sanitized by a typical circulation-cleaning treatment involving rinse, acid cleaner,[3] rinse, alkaline cleaner,[4] rinse, hypochlorite sanitizer,[5] and rinse, each for 5 min. at 140° F. Again, the white metal elbow was removed and replaced by the original stainless steel elbow, and the same skimmilk recirculated through the system. Copper concentration increased to 0.187 p.p.m., an increase of 0.080 p.p.m.

Experiments similar to the above were conducted with smaller equipment, to determine the suitability of laboratory-scale experiments for a study of copper adsorption and removal. A trial involving the same steps as the pilot-scale experiment was conducted with a one-liter stainless steel beaker, using as copper source a white metal nut suspended in the beaker from a glass hook. Results were similar to those of the pilot experiment. As an additional step toward simplifying the experimental approach, copper sulfate solution was used as the source of copper for adsorption. The following experiment illustrates representative procedure and results.

A one-liter stainless steel beaker was freed of copper by treatment with nitric acid and rinsed thoroughly with double-distilled water. Copper sulfate solution (500 ml. containing 0.2 mg. of copper) was placed in the beaker and agitated

[3] Sulfamic acid, Grasselli Chemicals Department, E. I. du Pont, Wilmington, Delaware.

[4] Sodium metasilicate—42%, tetrasodium pyrophosphate—28%, sodium carbonate—23%, alkylaryl sulfonate (Naconal NR)—7%.

[5] Alkaline sodium hypochlorite solution: Clorox, The Clorox Co., Oakland, Calif.

continuously for 15 min. at 25° C. The solution was removed and the beaker was thoroughly rinsed (four times) with double-distilled water. Milk (500 ml.) was placed in the beaker and agitated for 15 min. at 45° C. Samples of the milk before and after treatment in the beaker were stored in a refrigerator. The treated sample had a strong oxidized flavor in 19 hr., but the control sample was not oxidized after four days. Results of copper analyses are presented (Table 1).

TABLE 1

Results of copper determinations for experiment to illustrate copper contamination of milk from stainless steel beaker

No.	Sample and treatment	Copper	
		(p.p.m.)	*(mg.)*
1.	Copper sulfate solution after 15 min. in beaker	0.208	0.103
2.	Original milk	0.029	0.0145
3.	Milk after 15 min. in beaker	0.178	0.089
4.	Increase in copper in milk	0.149	0.0745
5.	Nitric acid used to treat beaker after removal of milk	0.020	0.010
	Total copper recovered (No. 1, 4, 5)		0.1875
	Total copper in original solution		0.200
	Recovery = 93.8%		

Such preliminary experiments helped determine the conditions used in the following study of factors that influence adsorption of copper on stainless steel and its removal.

Removal of adsorbed copper. A standardized procedure was adopted to adsorb copper on the stainless steel beaker. Each 500-ml. portion of the solution used contained 0.2 mg. copper (theoretical, added as $CuSO_4 \cdot 5H_2O$) and enough alkaline sodium hypochlorite solution [5] to give about 200 p.p.m. available chlorine and to adjust pH to 9.3. The solution in the beaker was agitated 15 min. at room temperature and discarded, and the beaker was rinsed four times with double-distilled water.

Nitric acid, milk, and a chelating agent [6] were compared for effectiveness for removing adsorbed copper (deposited as just described). In each case, the beaker was treated 5 min. with the test material and rinsed four times with double-distilled water, and any residual copper was removed by treatment with 500 ml. of hot (60–80° C.) 1.5 *N* nitric acid. Data are presented for both adsorption and removal of copper (Table 2).

Hot 1.5 *N* nitric acid was very effective for removing adsorbed copper from stainless steel. As a standard procedure in this study, all stainless steel and glass equipment was treated with hot nitric acid before use.

Adsorbed copper that was not removed by a thorough rinse with water was picked up fairly rapidly by milk, presumably because of milk's metal-binding properties. During 5-min. exposures, milk removed 77.6% of the adsorbed copper at 10° C., and 88.7% at 60°. The chelating agent tested was even more effective, giving 89.8% removal in 5 min. at 25° C. and 93.1% at 60°.

[6] Versene, the trisodium salt of ethylenediaminetetraacetic acid (EDTA), Bersworth Chemical Co., Farmingham, Massachusetts.

TABLE 2

Effectiveness of nitric acid, milk, and chelating agent (EDTA) for removal of adsorbed copper from stainless steel

No.	Removal agent	Temperature	Copper adsorption				Copper removal				
			Added in solution	Removed in solution	Adsorbed[a]		Removed by agent	Removed by nitric acid	Total removed	Recovery of adsorbed copper	Recovery by agent[b]
		(°C.)	(mg.)			(%)	(mg.)			(%)	
1.	Nitric Acid, 1.5. *N*	60	0.197	0.102	0.095[c]	49.5	0.099	0.001	0.100	105.1	99.0
2.	Milk	10	0.197	0.152	0.045	22.8	0.0295	0.0085	0.038	84.3	77.6
3.	Milk	60	0.197	0.146	0.051	25.8	0.0435	0.0055	0.049	96.0	88.7
4.	EDTA	25	0.197	0.144	0.053	26.8	0.040	0.0045	0.0445	83.8	89.8
5.	EDTA	60	0.197	0.138	0.059	30.0	0.0405	0.003	0.0435	78.5	93.1

[a] By difference, copper added less that remaining in solution.

[b] Based on total removed.

[c] Much higher than for the No. 2-5, possibly due to experimental error in pretreatment of the beaker, but this error would not influence conclusion regarding per cent recovery based on total copper recovered.

Influence of pH and composition of solution on adsorption. Experiments were conducted to determine the influence on copper adsorption of the pH and composition of the copper solution. The procedure was similar to that followed in previous experiments. Adsorbed copper was removed by hot 1.5 *N* nitric acid.

The results (Table 3) illustrate the important role of pH in determining the amount of copper that adsorbs. Maximum adsorption occurred in the pH range 8–9, with an appreciable amount over the range 6–10. The presence of hypochlorite markedly increased the amount of copper adsorbed. There was little adsorption at low pH either with hydrochloric or with sulfamic acid.

Influence of temperature on adsorption. The amount of copper that adsorbed on the stainless steel beaker was greater at 60° C. than at 25° C. (as illustrated in Table 4). The differences in amounts adsorbed were related to pH and composition of the solution, and consistent with results in Table 3.

Influence of initial copper concentration and time for adsorption. As shown in Table 5, the total amount of copper that adsorbed increased with an increase

TABLE 3

Influence of pH and composition of solution on adsorption of copper on stainless steel

Solution—copper sulfate and:	pH	Copper remaining in solution[a]	Copper adsorbed	Total recovered	Adsorbed[b]
		(mg.)			*(%)*
Sulfamic Acid	4	0.212	0.004	0.216	1.9
Hydrochloric Acid	4	0.199	0.004	0.203	2.0
Sulfamic Acid	5	0.207	0.007	0.214	3.3
Hydrochloric Acid	5	0.206	0.009	0.215	4.2
Sulfamic Acid	6	0.149	0.071	0.220	32.3
Hydrochloric Acid	6	0.134	0.069	0.203	33.9
No addition	7	0.125	0.089	0.214	41.6
Alkaline hypochlorite	8	0.077	0.132	0.209	63.1
Sodium hydroxide	8	0.090	0.116	0.206	56.4
Alkaline hypochlorite	9	0.103	0.107	0.210	51.0
Sodium hydroxide	9	0.136	0.068	0.204	33.3
Alkaline hypochlorite	10	0.123	0.083	0.206	40.3
Sodium hydroxide	10	0.164	0.031	0.195	15.7

[a] Initial copper in solution—0.2 mg. in 500 ml.; 15 min. at 25° C. allowed for absorption.
[b] Based on total recovered.

TABLE 4

Comparison of copper adsorption on stainless steel at 25° C. and 60° C.

Temperature	Solution—copper sulfate and:	pH	Copper remaining in solution[a]	Copper adsorbed	Total recovered	Adsorbed[b]
(° C.)			*(mg.)*			*(%)*
25	Sulfamic acid	2	0.106	0.004	0.110	3.8
60	Sulfamic acid	2	0.113	0.003	0.116	2.6
25	No addition	7	0.036	0.0655	0.1015	64.7
60	No addition	7	0.025	0.074	0.099	74.7
25	Alkaline hydrochlorite	10.3	0.093	0.011	0.104	10.9
60	Alkaline hydrochlorite	10.3	0.0455	0.0555	0.101	54.9
25	Sodium hydroxide	11.2	0.082	0.0155	0.0975	15.9
60	Sodium hydroxide	11.2	0.0775	0.0215	0.009	21.6

[a] Initial copper in solution—0.1 mg. in 500 ml., 15 min. allowed for adsorption.
[b] Based on total recovered.

TABLE 5

Influence of copper concentration and time for adsorption on amount of copper adsorbed

Initial copper concentration	Time for adsorption	Copper remaining in solution	Copper adsorbed	Total recovered	Adsorbed[a]
(mg/500 ml.)	*(min.)*		*(mg.)*		*(%)*
0.1	15	0.0535	0.0485	0.102	47.7
0.2	15	0.139	0.0575	0.1965	29.3
0.3	15	0.217	0.077	0.294	26.3
0.4	15	0.309	0.091	0.400	22.7
0.5	15	0.413	0.095	0.508	18.7
0.2	2	0.172	0.016	0.188	8.5
0.2	5	0.170	0.038	0.208	18.3
0.2	10	0.148	0.0525	0.2005	26.2
0.2	15	0.139	0.056	0.195	28.9
0.2	20	0.130	0.0725	0.2025	35.8

[a] Based on total recovered.

in concentration of copper in solution, but the percentage of the total copper that adsorbed decreased. Similarly, the total amount of adsorbed copper continued to increase for at least 20 min., but the rate of absorption decreased. Half of the copper that adsorbed in 20 min. had adsorbed during the first 5 min.

Influence of sanitizing agents on corrosion of white metal and adsorption of copper. In equipment containing both copper-nickel alloy and stainless steel, the amount of copper adsorbed on stainless steel presumably would depend both on the amount of copper dissolved from the alloy and on the suitability of conditions for adsorption. Experiments were conducted to determine the influence of selected sanitizing agents on corrosion and adsorption together, and on adsorption of copper from copper sulfate solution. For the experiments that depended on corrosion of copper-nickel alloy as the source of copper, a white metal sanitary nut was suspended in the stainless steel beaker by a glass hook. The sanitizing agents used were an alkaline cleaner,[4] sulfamic acid,[3] alkaline sodium hypochlorite solution,[5] a chelating agent (EDTA), a quaternary ammonium compound,[7] iodine sanitizer A,[8] and iodine sanitizer B.[9] The experimental conditions and results are tabulated (Table 6).

Of the sanitizers studied, sulfamic acid at 60° C. was the most corrosive to the copper-nickel alloy, followed by the alkaline cleaner, the iodine sanitizers at 25° C., and the hypochlorite at 60° C. Least corrosive were the sodium hypochlorite at 25° C. and the quaternary ammonium compound.

Copper adsorption was negligible with sulfamic and with the hypochlorite in the presence of EDTA. Adsorption was greatest with the hypochlorite at 60° C.

[7] Hyamine 1622 (*p*-diisobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride), Rohm and Haas Co., Philadelphia 5, Pennsylvania.

[8] Heliogen, a diatomic iodine sanitizer (Potassium iodide—27%, Chloramine-T—20%), S. B. Penick & Co., New York.

[9] Wescodyne, an iodophor (polyethoxy ethanol—iodine complex—7.75%, nonyl phenyl ether of polyethylene glycol—iodine complex—3.75%), West Disinfecting Co., Long Island City, New York.

TABLE 6

Influence of various sanitizing agents on corrosion of copper-nickel alloy and adsorption of copper on stainless steel

No.	Solution and concentration	Temperature	pH	Copper from corrosion of copper-nickel alloy				Copper added as copper sulfate[a]			
				Remaining in solution	Adsorbed[b]	Total corrosion	Adsorbed	Remaining in solution	Adsorbed[b]	Total recovered	Adsorbed
		(° C.)		(mg.)			(%)	(mg.)			(%)
1.	Alkaline cleaner, 0.25%	60	10.8	0.892	0.042	0.934	4.51	0.0775	0.0215	0.099	21.6
2.	Sulfamic acid, 0.25%	60	2.1	3.510	0.007	3.517	0.00	0.113	0.0025	0.116	2.2
3.	Sodium hypochlorite, 200 p.p.m. chlorine	60	9.3	0.254	0.114	0.368	31.0	0.072	0.114	0.186	61.3
4.	Sodium hypochlorite, 200 p.p.m. chlorine	25	9.3	0.033	0.0185	0.0515	36.1	0.139	0.056	0.195	28.9
5.	Sodium hypochlorite, 200 p.p.m. chlorine, plus EDTA, 0.25%	25	9.5					0.292	0.000	0.292	0.0
6.	Iodine sanitizer A, 125 p.p.m. iodine	25	7.6	0.523	0.0235	0.5465	4.3	0.180	0.009	0.189	4.8
7.	Iodine sanitizer B, 75 p.p.m. iodine	25	6.6	0.299	0.0395	0.3385	11.7	0.141	0.0325	0.1735	18.5
8.	Quaternary ammonium compound, 450 p.p.m.	25	8.1	0.0365	0.0095	0.046	20.3	0.130	0.049	0.179	27.3

[a] No. 1 and 2—0.1 mg.; No. 5—0.3 mg., and others—0.2 mg. per 500 ml.

[b] Determined by removal with nitric acid.

Adsorption was intermediate with the alkaline cleaner, hypochlorite at 25° C., iodine sanitizer B, and the quaternary ammonium compound.

DISCUSSION

For many years, it has been recognized that the first milk that passes through equipment after cleaning and sanitizing treatments is most heavily contaminated with metals. Explanations proposed for the greater initial contamination are based on the formation during runs of a protective coating that retards solution of the metal (2, 4), and on the formation between runs of a film of soluble metal oxides that is easily removed by the first milk (12). Results reported above indicate that copper adsorbed on stainless steel during sanitizing treatments would also contribute to the greater contamination early in processing.

The principal objective of this study was to provide information of help in preventing copper contamination of milk. The results demonstrate that a small amount of copper-bearing alloy in dairy equipment or in its cleaning circuit could cause disproportionately large copper contamination of milk, because of the amount of solubilized copper that might adsorb on stainless steel during cleaning. The obvious solution is to eliminate all copper-nickel alloys from dairy equipment.

Where copper-nickel alloys are used, approaches are indicated for minimizing contamination from adsorbed copper. The detergent selected for cleaning should cause as little corrosion of white metal as possible, thereby preventing copper from becoming available for adsorption on stainless steel. Conditions provided during circulation cleaning, particularly the final stages, should be selected to minimize adsorption of copper on stainless steel. Copper adsorption was slight or absent at low or very high pH, in the presence of a chelating agent, or with very low copper concentration. Hypochlorite sanitizer increased copper adsorption. If the conditions used for part of the cleaning process permit copper adsorption, a treatment that removes adsorbed copper, such as by a noncorrosive acid or a chelating agent, may be included near the end of the sanitizing procedure. If excessive copper contamination occurs from adsorbed copper, some of the first milk processed in the equipment may be discarded to reduce contamination of the entire supply.

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EVALUATING THE NUTRITIVE QUALITY OF FORAGE ON THE BASIS OF ENERGY. A REVIEW[1]

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The requirement of the animal for energy exceeds all of its other nutrient needs combined. It is obvious, therefore, that in feeding practice the adequacy of the energy supply must receive primary consideration. It is equally obvious that a knowledge of the nutritive value of feeds as sources of energy is essential. When it is recognized that over 75% of the feed consumed by ruminants comes from forages, it becomes apparent that a simple yet accurate measure of the energy value of this group of feedstuffs is one of the greatest needs in the field of ruminant nutrition.

Data presented by a number of workers (8, 35, 57) clearly illustrate the discrepancies which may occur when various methods are employed to assess the energy value of forages. For example, Blaxter (8) compared some of the feed-evaluation systems, using artificially dried immature grass and mature rye grass hay. The superiority of the immature dried grass varied from 29% according to the total digestible nutrient (TDN) system, to 86% when the feeds were evaluated according to the Fodder Unit System. These data illustrate that the assessment of energy in forages is an important problem and one demanding increased research attention.

CATEGORIES OF ENERGY

The starting point in estimating the energy value of a feed is the determination of its gross energy content. This represents the heat given off when the feed is completely burned in a bomb calorimeter to its ultimate oxidation products. As a measure of its nutritive value, the gross energy content of a feed is of no value. This is true because, in the processing of the feed by the animal, variable quantities of energy are lost in the form of combustible gases, feces, urine, and heat.

Deducting the energy lost in the feces from the gross energy gives the digestible energy (DE) content of the feed. This quantity of energy, excluding that contained in the fermentation gases, is absorbed from the gastrointestinal tract and is processed further, resulting in losses by way of the urine. The energy remaining in the animal's body after accounting for the losses in the feces, gas, and urine is referred to as metabolizable energy (ME). This is the energy available to the animal for various body functions, such as maintenance, weight gain, milk production, and work. However, not all of this energy is used for such functions since, in the utilization of the ME, a part of it is lost as waste heat.

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That portion of the ME remaining after subtraction of the heat loss is known as net energy (NE). This represents that portion of the gross energy which actually appeared as body tissue gained, milk secreted, or work performed.

EXTENT OF ENERGY LOSSES AND FACTORS DETERMINING THEIR SIZE

Methane. The amount of energy which is lost as methane may represent from 4 to 8% of the gross energy ingested as forage (9, 11, 21), and the amount decreases with increasing intake (9, 11, 21, 24). Since the production of methane is correlated with the intake of digestible carbohydrates, forages generally lose less energy by this means than do concentrates or mixed rations. More energy appears to be lost as methane when long hay is fed than when the same hay is ground and pelleted (9, 11).

Loss as heat of fermentation. This is the energy which is lost as a result of microbial digestion. It is included in the total heat loss, and although no completely satisfactory means is available for estimating this loss (39), separate from the heat arising from other metabolic processes, it would appear to represent approximately 10 to 15% of the total heat increment (1, 2, 41). On the basis of the results of Marston's (44) in vitro fermentation studies, Armstrong *et al.* (5) estimated that the heat of fermentation in sheep fed dried grass amounted to about 11% of the total energy absorbed (gross dietary energy minus the energy of feces and methane). Reid (56) states that from 1.5 to 11% of the gross energy of mixed rations is lost as fermentation heat.

Urinary loss. The amount of energy lost in the urine is of the same proportion as the methane loss, representing about 4–6% of the gross energy intake (9, 11, 21). The loss of energy in this form tends to be greater when the ration is high in nitrogenous compounds and the nitrogen is highly digestible. This is because the catabolism of protein in the animal body results in the formation of urea as well as other incompletely oxidized products which are excreted in the urine. With mixed rations there is a tendency for the loss of energy in the urine to decrease as the total feed intake increases (21, 24, 48). However, Blaxter and Graham (9, 11) found that with sheep fed artificially dried hay the urinary energy loss increased with increasing intake. They further observed (11) that the loss was significantly higher with long hay than with ground and pelleted hay.

The losses of energy in the feces and as heat are discussed in other sections of this paper.

FACTORS AFFECTING THE UTILIZATION OF METABOLIZABLE ENERGY

As previously stated, the utilization of ME results in the loss of variable amounts of energy in the form of heat. Subtraction of the heat loss from ME leaves NE, which is that energy which is reflected in maintenance, body gain, and milk production. Of the many factors known to influence the extent of the heat loss, the most important are: *(1)* the level of feed intake, *(2)* the balance of nutrients in the ration, *(3)* the end products of digestion, and *(4)* the physiological function for which the ME is used.

Level of feed intake. That the amount of energy lost as heat (and, therefore, the net availability of ME) varies with feed intake has been shown in a number of studies with cattle (21, 24, 48) and sheep (9, 11, 41), as well as with other animals (8). All of these studies demonstrate that the relationship between feed intake and energy retention is curvilinear but, as pointed out by Blaxter (8), "differences of opinion have arisen as to whether the relationship can not be taken to be curvilinear below maintenance and rectilinear above." To be sure, the curvature of the line is much greater below energy equilibrium (maintenance) than it is above this level. Thus, the extent to which the net availability of ME is influenced by the level of feed intake depends on whether the fasting level or the maintenance level is used as the base line. For example, Blaxter (7) and Reid (56) have calculated from the data of Forbes *et al.* (21, 24) that, when fasting heat production is employed as the base line, the net availability of the ME of the same ration for fattening by cattle ranged from about 85 to 63%, when the level of intake was increased from that of maintenance to that of three times maintenance. On the other hand, when the heat production of maintenance was used as the base value, the net availability of ME above the maintenance level for the same range of intakes was 62 to 55%. Even though these calculations indicate that the relationship between the utilization of ME and feed intake is essentially rectilinear above energy equilibrium, Blaxter and Graham (9) are of the opinion "that to retain the linear system is to perpetuate gross errors."

Data cited by Blaxter (8) suggest that the nutritive value (net energy value) of some feeds decrease to a much greater extent with increasing intake than do others. Reid (56) and Huffman (36) both have suggested the use of early-cut forages to reduce the depressing effect of a higher level of feeding on the nutritive value (presumably net energy value) of the ration.

Balance of nutrients in the ration. The utilization of ME as influenced by dietary deficiencies and nutrient balance has been reviewed by Kleiber (37), Reid (56), and Blaxter (8). The early experiments of Kellner [cited by Blaxter (8)] demonstrated that the net energy value of feeds was decreased when the fiber content of the ration was raised. Mollgaard [cited by Blaxter (8)] found that the net energy value of feed for milk production was dependent on the protein content of the ration. A deficiency of phosphorus causes a reduction in the net availability of ME (38); whereas, a vitamin A deficiency has no effect (62). Heat increment is not affected by deficiencies of vitamin D (17) or magnesium (13); however, the basal heat production is increased and, thus, total heat production is raised when these two nutrients are limiting.

As a result of experiments (22, 23) in which it was found that the net energy value of corn grain was lower when fed alone than when it was fed in combination with hay, as well as other similar observations, Forbes (20) concluded that only when individual feeds are a part of "qualitatively complete and quantitatively sufficient" rations do they exhibit their optimal value. Mitchell (46) expressed the same idea, although more rigorously, when he postulated that "the net availability of the metabolizable energy of all perfectly balanced rations is maximal." As pointed out by Blaxter (8), the correctness of the view expressed

by Mitchell and implied by Forbes is dependent upon the definition of the term "balanced." Blaxter contends that if the term is used to describe rations optimal with respect to protein, minerals, and vitamins only, then the theories are not tenable, and he has criticized these concepts on the following bases: *(1)* The utilization of ME is not affected by a deficiency of the dietary essentials, vitamin A, vitamin D, and magnesium, *(2)* on the basis of Breirem's analysis [cited by Blaxter (8)] of data from Denmark, Germany, and the United States, the net availability of ME declines as the fiber content of the ration increases, and *(3)* the physical form of the ration and its water content influence the net availability of the energy it contains. With respect to physical form of the ration, Blaxter and Graham (10) reported in 1955 that, when sheep were given dried grass in the long form, the net availability of the ME it contained was lower than when the grass was ground and fed in pellet form. However, these workers later reported (11) that although the pelleting process did affect the distribution of energy losses (increased fecal loss, decreased heat loss), the net energy value of long dried grass was essentially the same as when the grass was ground and pelleted. This agrees with the earlier observations of Forbes *et al.* (26). The data of Blaxter and Graham (11) do indicate that the pelleted forage would be superior in nutritive value to the long forage at high levels of intake.

End-products of digestion. The view has been expressed by a number of workers (8, 36, 64) that the proper assessment of feeds as energy sources for ruminants rests on a thorough knowledge of the role of the reticulo-rumen in the over-all nutrition of the animal. As stated by Blaxter (8), "the key to food evaluation and a deeper understanding of the energy nutrition of the ruminant animal lies in the elucidation of the chemical, physical, and biological factors that control the bacterial populations of the rumen and the kinetics and energetics of the subsequent dissimilation of the products arising from bacterial fermentations of the complex carbohydrates of the ruminant's diet."

Numerous investigations (6, 14, 31, 51, 64, 68, 69, 70) have demonstrated that the nature of the diet influences the proportion of the volatile fatty acids produced in the digestion of carbohydrates by cattle, sheep, and goats. In 1864, Grouven [cited by Blaxter (8)] attributed the high heat increment of the ruminant to the metabolic stimulus of the acids produced during microbial fermentation in the rumen. Marston (43) and McClymont (42) also have suggested that the intermediary metabolism of fatty acids, particularly acetic acid, is the main factor accounting for the high heat increment in the ruminant. The recent studies of Armstrong and coworkers (3, 4, 5) have added materially to our knowledge of the contribution of the steam-volatile acids to the heat increment of the ruminant animal. It was shown (3) that when acetic, propionic, and butyric acid were administered into the rumen of fasting sheep, the heat increments of the acids (as per cent of acid metabolized) were 41, 13, and 16%, respectively. In subsequent experiments (5), the heat increments (13 to 17%) which resulted from the administration of various mixtures of the acids were considerably lower than the expected heat increments based on the separate effects of the acids. Thus, it was shown that the metabolism of acetic acid was

greatly modified by the presence of small quantities of propionic and butyric acids, and that it is incorrect "to consider the metabolism of each acid separately in order to find the cause of the high heat increment of the ruminant animal" (3). The efficiency with which the acids are used to effect an increase in body tissue is considerably less than when they are utilized for maintenance purposes (4, 5). As pointed out by Blaxter (8), it is indicated that differences in the ratios of fatty acids absorbed represent, in effect, the associative effects of feeds which influence quite markedly the net availability of ME.

In the normal ruminant the heat increment associated with the metabolism of starch is twice as high as in nonruminants (4). However, Armstrong *et al.* [cited by Reid (58)] found that a sheep whose rumen had been flushed out and treated with penicillin, to minimize microbial fermentation, utilized for maintenance the energy provided by glucose just as efficiently as did simple-stomached animals. In such sheep, the energy from glucose was used for maintenance more efficiently than the energy from volatile fatty acids. Thus, it is apparent that the type of end-product produced from carbohydrate degradation has a marked effect on the heat increment and, therefore, on the net availability of ME. These are interesting observations in the light of the work of Matrone *et al.* (45), who found that lambs grew better on a purified diet containing the salts of acetic, propionic, and butyric acids than on diets containing either starch and glucose or starch, glucose, and cellulose.

Function for which the ME is used. If it is assumed that the relationship between the level of feed intake and the retention of energy by the animal is curvilinear, then the value of a feed for the maintaining of energy equilibrium is greater than for increase of body tissue. The experiments of Armstrong and coworkers (4, 5) have shown that the volatile fatty acids are utilized much more efficiently for maintenance than for fattening. Fries *et al.* (30) and Forbes *et al.* (27) found, in studies with dairy cows, that the ME provided in excess of energy balance was used for milk production and weight gain, 98.5 and 76%, respectively, as efficiently as ME was used for maintenance. The mean value for the net availability of ME above the energy equilibrium level calculated by Reid (56), from the data reported by Kriss (39) and Forbes and Kriss (28), was 58% for fattening. Despite the varied experimental conditions imposed in these studies, the coefficient of variation between animals was quite low. Although not studied extensively, the availability of ME above energy equilibrium for milk secretion appears to be approximately 69% (56). Blaxter (8) and Armstrong and Blaxter (4) have discussed the possible reasons for the higher nutritive value of a feed as a source of energy for lactation or maintenance compared with fattening. Since depot and milk fat differ in fatty acid composition, one of the factors involved appears to be the increased energy cost of depot fat synthesis compared with milk fat synthesis.

It was in the light of data such as that cited above that Mitchell (46) based his view that the utilization of the ME of balanced rations is maximal for the same animal species and for the same body function. As pointed out previously, Blaxter (8) has offered some very credible criticisms of this concept. However,

as stated by Reid (58), "the apparent constancy with which cattle utilize ME for maintenance, body gain, and milk secretion is remarkable, at least with the usual rations fed."

Since the losses of energy as methane and urine are small, and if it is assumed that the amount of energy lost as heat is approximately the same for all balanced rations used for the same physiological function, then the most significant form of energy loss, because of its magnitude and variability, is that via the feces. Therefore, the simplest, most accurate measure (in terms of the mechanics involved) of the energy value of forages is that based upon the difference between the gross energy of the feed consumed and the feces voided.

Factors Affecting Energy Losses in Digestion

Although the heat of fermentation should logically be charged as a digestive loss, it will not be considered here, since it has been discussed elsewhere in this paper, and also because no completely satisfactory means is available for measuring this loss separate from the heat arising from other metabolic functions.

In the majority of farm animals, the major loss of dietary gross energy in practically all cases is that which is lost in the feces. This represents from 20 to 60% in ruminants consuming forage rations. A number of conditions, as reviewed by Colovos (16), are known to influence this energy loss. The following will be given consideration here: *(1)* level of feed intake and physical form of ration, *(2)* growth stage of forage, *(3)* associative effects of nutrients, and *(4)* species of animal and age of animal.

Level of feed intake and physical form of ration. A number of studies (56) have demonstrated that the extent to which level of intake affects the amount of dietary energy lost in the feces is dependent upon the type of ration consumed. It is a well-established fact that the fecal loss of energy from mixed rations and corn silage is markedly increased by increasing the intake. On the other hand, the digestibility of forages (exclusive of corn silage) fed in the long form is not materially affected by level of intake (12, 55, 56). Very few results with fresh herbage have been published.

Several recent studies (11, 12, 40, 63) have shown that the feeding of ground or ground and pelleted forage results in a considerably greater fecal energy loss than when the same forage is consumed in the long form. Most of this depression in digestibility appears to be attributable to a higher feed-intake level (11, 12). The grinding and pelleting process depresses primarily the digestibility of cell-wall constituents, and the decrease in the over-all digestibility of the forage brought about by such processing apparently is the result of the increased rate at which it passes through the gastrointestinal tract of the animal (12, 63). It might be reasoned that the physical nature of long or chopped forage prevents an intake sufficient to increase the passage rate, to the extent that digestibility would be greatly depressed.

Growth stage of forage. It has been known for some time that one of the major determinants of the nutritive value of forages is the stage of growth at

time of harvesting or grazing; however, it has been only within recent years that this important factor has been given particular attention in studies of forage evaluation. The studies of Reid and coworkers (56), covering a period of several years, as well as those of Homb (35) and Jarl and Helleday [cited by Reid (56)], demonstrate the significant influence of age of plant on fecal loss and show that the extent of the latter may be predicted from the former.

Associative effect of nutrients. When a combination of feeds is found to yield a digestibility value higher than that given when either feed is fed alone, this phenomenon is referred to as associative digestibility. As pointed out by Reid (58), even though this effect seldom if ever exceeds 10% of the digested energy, it indicates that the digestible matter or energy of individual feeds is not strictly additive and that the digestibility of a ration can not be predicted accurately from the digestibility of the individual components.

The digestibility of feeds low in protein is generally improved by the addition of high-protein feeds (32, 56). A deficiency of phosphorus (38), vitamin D (17), or magnesium (13) has no effect on the loss of energy in the feces; whereas, a deficiency of vitamin A (62) increases the loss. That some inorganic constituent(s) are important for optimum digestion is indicated by the work of Swift *et al.* (67) and others (71), in which it was found that the addition of alfalfa ash to certain high-fiber rations improved the digestibility of the rations by ruminants. Recent work (18) suggests that the beneficial effect of alfalfa ash may be due, in part at least, to its content of molybdenum. In 1942, Mitchell (47) pointed out the importance of the associative effects of feeds in digestion and suggested, partly as a result of his own work (49), that the effect is brought about by a modification of the activities of the rumen microorganisms. This same view was expressed by Forbes and Swift (29).

Swift (65) has emphasized the associative effect of feeding with reference to the nutritive evaluation of forages, and he concluded that, "It seems reasonable to assume that if we test forages by considering them to be 'complete rations,' we are on infinitely safer ground than would be the case if we add the unmeasurable complication of concentrate supplementation."

Species of animal and age of animal. Because of the labor and expense involved in digestion studies with cattle, the question arises as to whether the values obtained in such studies with sheep or even rabbits would be applicable to cattle. Forbes *et al.* (25) found that, with the exception of crude fiber, sheep digested mixed rations more efficiently than did steers. These workers suggested that published coefficients with any species should be based upon digestion trials conducted with that species. Later work by Watson *et al.* (72) indicated that the differences in the digestive abilities of sheep and cattle were not of very great magnitude. As a result of a statistical study of the published digestibility data, Cipolloni *et al.* (15) concluded that, "greater accuracy will be attained if digestibility data to be used for cattle are obtained with cattle, and similarly with sheep." Comparative digestion studies with sheep and cattle being conducted cooperatively among several northeastern states indicate that results

obtained with sheep are applicable to cattle, and vice versa (66). The usefulness of rabbits as tester animals in digestion studies appears to be limited (33, 61).

Since digestion in the ruminant depends on the establishment of a complex microbial population in the rumen and on the efficient mastication of fibrous feeds, it seems reasonable to expect that forage digestibility and age of the animal would be related. In sheep it has been found that digestibility increases about one percentage unit per year from lambs to 2-yr.-olds (54). However, Preston *et al.* (53) found that young calves digested the dry matter of fresh grass to the extent of 74.6% and that no variation in digestive ability was observed between 3 and 9 wk. of age. Also, McArthur (41) reported that 8 to 10-wk.-old calves digested high-quality grass as efficiently as did mature cows.

Since the secretion of thyroxine in the ruminant fluctuates with season (19, 34, 52), and since the number and/or activity of the ruminal microorganisms appears to exhibit seasonal variation (50), it seems reasonable to suspect that efficiency of digestion may be influenced by season of the year. Indeed, Raymond *et al.* (54) have presented evidence to suggest that the digestive efficiency of sheep fluctuates with season.

Criteria of Nutritive Value of Forage Based upon Digestibility

There are three measures of nutritive value in common usage which represent differences between the feed consumed and the feces voided. These are digestible dry matter (DDM) (or digestible organic matter), total digestible nutrients (TDN), and digestible energy (DE). In essence, all of these measure essentially the same thing—the proportion of ingested matter or energy that disappears as the feed traverses the gastrointestinal tract. Because of this fact, these criteria are highly correlated one with the other (56, 65). Also, there is a high correlation between them and ME (56, 65). As pointed out by Swift (65), except with grazed herbage where DDM is the criterion of choice, DE is the preferred measure of the nutritive value of forage. This is because it is more direct and accurate than TDN and because it is "free from empirical procedures and assumptions."

Since only one source of energy loss (namely, that in the feces) is accounted for in the determination of digestible energy, it is conceivable that this criterion would measure the nutritive value of feed with considerable error. The data of Blaxter and Graham (11) show this to be true. Although the biological response which a feed promotes (its NE value) is a much more accurate measure of the nutritive value of the feed than what it contains (its DE value), very few laboratories are equipped for the determination of complete energy balances. Also, as made by conventional means, such determinations are expensive, laborious, and quite limiting with respect to number of animals involved and experimental conditions. (It should be pointed out that the conventional slaughter experiment permits net energy estimates to be made without resort to a complete energy balance.) The indirect calorimetric method proposed by Reid and coworkers (59, 60), which circumvents the above-mentioned difficulties, would

appear to hold great promise for the assessment of the energy value of forages under grazing as well as stall-feeding conditions. This method involves the estimation of the chemical composition of the intact, live animal from a knowledge of the water content of the ingesta-free body. The application of the well-established caloric values of protein and fat to the contents of these two constituents allows the caloric value of the whole empty body to be determined. It remains to be seen what contribution such a procedure will make to the complex problem of forage evaluation.

Since, with the usual rations fed, relatively constant proportions of ME are put into such functions as milk production, maintenance, and body increase, a measure of digestible matter or energy is the best practical measure of the productive value of forages that can be obtained with the facilities in all laboratories. However, basic studies should be continued and initiated to develop new techniques for arriving at the NE value of forages and to determine the conditions which alter this value.

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SOME INTERRELATIONSHIPS BETWEEN VITAMIN E AND VITAMIN A IN HOLSTEIN CALVES[1]

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SUMMARY

Thirty-six Holstein male calves, 64 days of age and partially depleted of their tocopherol and vitamin A stores were fed, in addition to a basal ration low in tocopherol and vitamin A, one of three levels of tocopheryl acetate, equivalent to 1, 5, or 25 mg. of free tocopherol per pound live weight per day and one of three levels of vitamin A palmitate, equivalent to 10, 100, or 1,000 γ of vitamin A alcohol for a 4-wk. period. Upon completion of a 28-day tocopherol–vitamin A supplementation period, one-half of the calves were slaughtered and the other 18 calves were maintained on only the basal ration until plasma vitamin A decreased to 4.0 γ/100 ml. Based on plasma and liver vitamin A concentrations at the termination of supplementation and on vitamin A depletion time, tocopherol resulted in an increase in utilization of vitamin A at the highest intake of vitamin A, decreases at the lowest intake of vitamin A, and inappreciable change at the middle intake. Across all levels of tocopherol intake, the tocopherol concentration of the tissues decreased with increasing intake of vitamin A. This decrease was greater between the 10 and 100 γ intake of vitamin A than between the 100 and 1,000 γ intake. These results indicated a need to establish levels or ratios of intakes of vitamins A and E.

The sparing effect of tocopherol on vitamin A or carotene in vivo has, in general, been demonstrated with various laboratory animals, particularly the rat (5, 17, 18, 22). Early work (23) on the effect of tocopherol on the utilization of carotene and/or vitamin A by domestic animals was, for the most part, negative. However, recently in a single-reversal experiment with monozygotic-twin lactating cows grazing summer pasture (19), a marked increase in carotene and vitamin A concentration of the milk fat was observed when supplements of 1 g. of α-tocopherol were fed daily.

In possible contrast to the sparing action of tocopherol on carotene or vitamin A reported above, two groups of workers (10–12, 15, 31) indicated that when tocopherol, either in the esterified or free form, was fed in relatively high amounts to rats fed a fixed amount of carotene, decreased liver storage of vitamin A resulted. No such decrease in liver vitamin A storage was observed when vitamin A was fed in place of carotene or when 8 hr. elapsed between the feeding of tocopherol and carotene. In a recent study (25), in which calves, lambs, and pigs were fed graded levels of α-tocopheryl acetate and one level of vitamin A, slight increases in plasma vitamin A concentration with increasing

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tocopherol intake were observed, accompanied by slight decreases in liver vitamin A concentrations.

The present study was undertaken to investigate further the effects of tocopherol on the utilization of vitamin A by Holstein calves, employing plasma and liver concentrations of vitamin A and vitamin A depletion time as criteria. The study also included the possible effects of vitamin A on the plasma and tissue concentrations of tocopherol and the change in plasma tocopherol concentrations during subsequent vitamin A depletion and plasma and liver tocopherol concentrations at the termination of vitamin A depletion.

EXPERIMENTAL PROCEDURE

Animals and rations. Thirty-six one-day-old Holstein male calves, obtained from various Connecticut State institution herds during August–September, 1956, were placed in individual tie stalls at the University research barn. Each calf either nursed its dam for approximately a 24-hr. period or received 8.0 lb. of colostrum. Thereafter, calves were fed limited whole milk, limited starter, and ad libitum U.S. No. 2 mixed hay (chopped) through the 35th day of age. On the 36th day of age, each calf was fed a vitamin A depletion ration, which was also low in total tocopherol content (25), at intakes to provide an anticipated live weight increase of 10 lb. per seven-day period. In addition to the vitamin A depletion ration, each calf received 6.0 γ of vitamin A from a dry carrier (NOPCAY "10" Type IV, 10,000 U.S.P. units per gram) per pound of live weight per day.

On the 64th day of age, each calf was fed, in addition to the depletion ration, one of three levels of tocopherol from a dry carrier (American Cyanamid Co. vitamin E feed supplement, Lot 0118, 20,000 I.U. vitamin E per pound, a concentrate of D-α-tocopheryl acetate absorbed on solvent-extracted soybean flakes), at intakes equivalent to 1.0, 5.0, or 25.0 mg. of free tocopherol per pound live weight per day and one of three levels of vitamin A from a dry carrier (NOPCAY 250 Type M, Lot V8339, 250,000 U.S.P. units per gram, a concentrate of synthetic vitamin A palmitate absorbed on soybean and wheat germ meals and stabilized with ethylcellulose), at intakes equivalent to 10, 100, or 1,000 γ of free vitamin A (alcohol) per pound live weight per day. To facilitate weighing supplement for those calves fed the 10 and 100 γ intakes, the potency of the dry carrier of vitamin A was reduced to 10,000 U.S.P. units of vitamin A per gram by premixing with an appropriate quantity of finely ground depletion ration. The first nine calves to arrive at the research barn were randomly assigned to one of the nine treatments as described above and this procedure was repeated for a total of four groups of nine calves each. Upon completion of four consecutive seven-day periods of tocopherol–vitamin A supplementation (92nd day of age), each calf in the first and third groups was slaughtered. The remaining calves were maintained on the vitamin A depletion ration without vitamin A or tocopherol supplementation until each calf's blood plasma vitamin A concentration decreased to less than 4.0 γ per 100 ml. for two consecutive seven-day periods, to determine vitamin A depletion time (14).

Calculation of the amount of tocopherol and of vitamin A to be fed to each calf, incorporation into the vitamin A depletion ration, and treatment for scours were identical to procedures previously used at this station (26).

Light exposure was limited to incandescent light from 6 A.M. to 6 P.M. daily and averaged during tocopherol–vitamin A supplementation 10.2 foot-candles with a standard error of 1.9. Average minimum and maximum daily temperatures during the same period were, respectively, 55.6 ± 0.3° F. and 65.6 ± 0.3° F.

Observations and analyses. The daily allowance of the vitamin A depletion ration was weighed to the nearest 0.1 lb. and the tocopherol and vitamin A supplements to the nearest 0.01 g. Live weights of each calf were recorded at successive seven-day age intervals throughout the entire experiment. Venous blood samples were obtained by jugular puncture on the 63rd and 91st days of age and at successive seven-day intervals thereafter for those calves maintained on the vitamin A depletion ration, for the purpose of determining vitamin A depletion time. The concentration of total tocopherol in the plasma was determined by saponification of the plasma, Swick and Baumann (30), prior to extraction with Skellysolve-B and subsequent analysis by the method of Quaife and Harris (24). Plasma carotenoids and vitamin A were determined by the Kimble procedure (16). The concentrations of carotenoids in the plasma were inappreciable, due to the low carotene content of the ration, and are not reported herein.

In the case of calves slaughtered at the completion of the tocopherol–vitamin A supplementation period, samples of liver, heart, trapezius muscle, and perinephric fat were obtained and held at 0° F. Vitamin A (and carotenoids but not reported) was determined on the livers by a modification of the Gallup-Hoefer method (3) and lipids and total tocopherol on all tissues, as previously described (7, 25).

In the case of calves fed the vitamin A depletion ration after completion of tocopherol–vitamin A supplementation, spinal fluid pressure (20) was determined on each calf during the second seven-day period after the blood plasma vitamin A level had decreased to less than 4.0 γ per 100 ml. Upon the completion of the above second seven-day period, each calf was slaughtered. At this time, the liver was removed, ground, sampled, and stored at 0° F. for total tocopherol analysis.

Samples of the starter, mixed hay, tocopherol, and vitamin A supplements were obtained at 4-wk. periods and analyzed for proximate constituents by A.O.A.C. procedures (2) (Table 1). Tocopherol content in the dry carrier was determined by refluxing 0.5 g. of sample in 75 ml. of purified Skellysolve-B for ½ hr., cooling to room temperature, transferring to a 250-ml. volumetric flask, making up to volume with Skellysolve-B, and allowing to stand at room temperature for 1 hr. A 10-ml. aliquot was then saponified and chromatographed, Eggitt and Ward (8), and the Emmerie-Engel reaction read at the end of 15 sec., as in the method of Quaife and Harris (24). Four such determinations, during the course of tocopherol–vitamin A supplementation, averaged 33.4 mg. of free tocopherol per gram, with a standard error of 0.9. Analyses of the vitamin A dry carrier (1) averaged 72.4 ± 0.6 mg. of vitamin A alcohol per gram.

TABLE 1

Average chemical composition of feeds and supplements

	Dry matter	Dry matter					
	Dry matter	Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	Carotene
	(%)						(mg/lb)
Calf starter	91.0 ± 0.3[a]	18.6 ± 0.6	3.7 ± 0.4	9.6 ± 0.6	59.5 ± 0.4	8.6 ± 0.8	0.22 ±0.08
Mixed hay, chopped	90.7 ± 0.9	10.5 ± 0.5	2.0 ± 0.2	31.2 ± 1.3	48.6 ± 0.6	7.7 ± 0.3	7.29 ±1.37
Vitamin A depletion ration	90.0 ± 0.4	15.7 ± 0.3	2.4 ± 0.1	13.8 ± 0.4	61.1 ± 0.3	7.0 ± 0.3	0.06 ±0.02
Tocopherol supplement	90.6 ± 0.8	51.3 ± 0.3	10.9 ± 0.2	2.9 ± 0.2	28.8 ± 0.3	6.1 ± 0.1	.....
Vitamin A supplement	93.0 ± 0.7	13.7 ± 0.9	44.1 ± 1.6	17.4 ± 0.9	22.8 ± 1.5	2.0 ± 0.1	.....

[a] Standard error.

Statistical procedures as outlined in Snedecor (29) and Cochran and Cox (4) were used in the analyses of the data. The reasons for the transformation of the liver vitamin A and tocopherol concentrations to their respective logarithms (Base 10) and the functional relationships between intake and concentration of plasma and liver vitamin A or tocopherol have been previously discussed (25, 27).

RESULTS

Feed consumption, growth, and health. In general, calves readily consumed their entire allowance of depletion ration plus tocopherol–vitamin A supplements. Average percentage days without feed refusals was 96.8, with a standard error of 0.8. A slight increase in these percentages was observed with increasing tocopherol intake, but this increase was found not to approach statistical significance. Percentage days free of scours was 95.1 ± 1.1, and the square root of the number of treatments for scours plus one-half was 0.76 ± 0.03, with neither criterion being affected by tocopherol or vitamin A intake.

The average live weight the day prior to tocopherol–vitamin A supplementation was 175 lb., with a standard error of eight, and the gain during the four seven-day periods of supplementation averaged 43 ± 1 lb. The average seven-day period gain in live weight during the vitamin A depletion period was 13.3 ± 0.9 lb.

Vitamin A. In Table 2, plasma and liver concentrations of vitamin A at the termination of tocopherol–vitamin A supplementation are given. Vitamin A depletion time, rates of decrease of plasma vitamin A, and terminal spinal fluid pressures for vitamin A depletion calves are presented (Table 3).

Across all levels of intake of tocopherol, plasma vitamin A concentration (adjusted values) was found to increase by 16.0 ± 1.1 γ/100 ml. for each log unit increase in intake of vitamin A, and liver vitamin A by 1.28 ± 0.05 log γ/100 g.,

TABLE 2

Effect of tocopherol–vitamin A supplementation on vitamin A tissue concentration at the termination of 4 wk. of supplementation

Vitamin A[a] intake	Tocopherol[a] intake	Plasma vitamin A[b]			Liver[c]	
		Initial	Terminal	Adjusted[d] terminal	Lipids	Vitamin A
(γ)	*(mg.)*	*(γ/100 ml.)*			*(g/100 g.)*	*(log [γ/100 g.])*
10	1	11	17	18	3.79	2.42
10	5	12	15	15	3.88	2.26
10	25	16	18	16	3.93	2.25
100	1	15	32	30	4.06	3.87
100	5	13	32	32	3.91	3.76
100	25	11	30	31	3.92	3.76
1,000	1	14	43	42	3.95	4.84
1,000	5	12	48	49	4.10	4.91
1,000	25	10	53	55	4.24	4.86
Standard deviation of a calf		3	6	6	0.16	0.17

[a] Intake per pound live weight per day.
[b] Values represent four calves per treatment group.
[c] Values represent two calves per treatment group and are expressed on a wet basis.
[d] Adjusted by covariance for initial plasma vitamin A values of the day prior to tocopherol–vitamin A supplementation.

with slight negative curvature existing in the last-named estimate. Across all levels of intake of vitamin A, tocopherol caused an increase of plasma vitamin A concentration of 2.6 ± 1.6 γ/100 ml. for each log unit increase in intake of tocopherol. This relationship, however, was not consistent with increasing intake of vitamin A, as evidenced by a significant interaction of the linear trend of vitamin A intake times the linear trend of tocopherol intake ($P < 0.05$). Tocopherol depressed plasma vitamin A concentration, −1.4 γ per log unit intake of tocopherol, at the 10 γ level of vitamin A intake; exhibited inappreciable effect, + 0.3 γ, at the 100 γ intake; and increased the concentration, + 8.8 γ, at the

TABLE 3

Effect of tocopherol–vitamin A supplementation on vitamin A depletion

Vitamin A[a] intake	Tocopherol intake[a]	Vitamin A depletion[b]			
		Vitamin A depletion time		Rate of change of plasma vitamin A	Terminal spinal fluid pressure
		Actual	Adjusted[c]		
(γ)	*(mg.)*	*(wk.)*		*(γ/100 ml/7 days)*	*(log [mm. H_2O])*
10	1	4	5	−2.56	2.02
10	5	4	4	−1.40	2.10
10	25	4	2	−2.26	2.02
100	1	15	13	−1.87	2.35
100	5	12	12	−1.87	2.17
100	25	14	15	−1.70	2.20
1,000	1	28	28	−0.87	2.27
1,000	5	34	34	−0.56	2.29
1,000	25	40	41	−0.58	2.30
Standard deviation of a calf		3	3	0.19	0.16

[a] Intake per pound live weight per day.
[b] Values represent two calves per treatment group.
[c] Adjusted by covariance for initial plasma vitamin A values of the day prior to tocopherol–vitamin A supplementation.

1,000 γ intake. Somewhat similar findings applied to liver vitamin A concentrations, i.e., tocopherol decreased liver vitamin A storage expressed as log γ/100 g. at the lowest vitamin A intake, caused smaller decreases at the intermediate vitamin A intake, and resulted in an inappreciable or a slightly positive change in storage at the highest vitamin A intake. These changes with respect to the effect of tocopherol on liver concentration of vitamin A did not approach statistical significance (at $P < 0.05$); however, it seemed desirable to point out the trends because of their general agreement with plasma vitamin A and vitamin A depletion time.

The livers weighed 1,651 ± 151 g. per calf, averaged 26.8 ± 0.9 g. of dry matter per 100 g., and showed an increased lipid concentration of 0.12 ± 0.05 g/100 g. for each log unit increase in intake of vitamin A. An increase in lipid concentration of the livers of 0.07 ± 0.07 g/100 g. for each log unit increase in intake of tocopherol, was also observed, but this did not approach statistical significance. Expressing liver vitamin A concentration on a lipid basis resulted in no appreciable change in the corresponding relationships of concentration either to vitamin A or to tocopherol intake.

Similar to the trends for plasma vitamin A and log liver vitamin A concentrations, vitamin A depletion time (Table 5) increased with increasing intake of vitamin A, by an amount of 15.5 ± 0.8 wk. for each log unit increase in vitamin A intake (adjusted vitamin A depletion time values), with slight positive curvature existing in this estimate ($P < 0.01$). Tocopherol, across all vitamin A intakes, increased vitamin A depletion time by 2.8 ± 1.1 wk. for each log unit increase in tocopherol. At the lowest vitamin A intake level, tocopherol caused a slight decrease in vitamin A depletion time of − 1.9 wk. per log unit increase in intake of tocopherol; at the 100 γ intake of vitamin A, little change, + 0.8 wk., in vitamin A depletion was observed; and at the 1,000 γ intake of vitamin A, a marked increase of + 9.6 wk. was found. While the rates of decrease of plasma vitamin A per seven-day period during vitamin A depletion appeared to exhibit slight positive change due to previous tocopherol intake, i.e., the rates of decrease, in general, were of smaller magnitude at the higher tocopherol intakes than at the lower intakes, statistical analysis indicated that the differences were only attributable to the previous intake of vitamin A. Spinal fluid pressures (expressed as log of the mm. H_2O and taken at the termination of vitamin A depletion when the blood plasma vitamin A concentration for each calf had decreased to less than 4.0 γ/100 ml. for two consecutive seven-day periods) did not appear to be associated with previous tocopherol intake. Although spinal fluid pressure did increase with previous vitamin A intake, undoubtedly this was due to the length of time on vitamin A depletion, as has been observed previously (6), rather than to previous vitamin A intake per se.

Tocopherol. In Table 4 are given total tocopherol concentrations of plasma, liver, heart, trapezius muscle, and perinephric fat. Tissue dry matter and lipid concentrations in g/100 g. for the last three tissues were, respectively, 20.3 and 2.4, 22.9 and 1.1, and 82.0 and 77.4, with standard deviations, respectively, of 0.5 and 0.1, 0.9 and 0.1, and 4.4 and 4.3. Neither dry matter nor lipid concen-

trations of these three tissues showed consistent or significant change with increases in tocopherol or vitamin A intake.

Across all levels of vitamin A intake, the tissue concentrations of tocopherol exhibited positive linear rates of change on tocopherol intake. Plasma tocopherol increased 253 ± 25 γ/100 ml. per log unit intake of tocopherol; liver, 0.54 ± 0.03

TABLE 4

Effect of tocopherol–vitamin A supplementation on tocopherol tissue concentration at the termination of 4 wk. of supplementation

Vitamin A intake[a]	Tocopherol intake[a]	Plasma tocopherol[b]		Liver tocopherol[c]	Heart tocopherol[c]	Trapezius muscle tocopherol[c]	Perinephric fat tocopherol[c]
		Initial	Terminal				
(γ)	*(mg.)*	*(γ/100 ml.)*		*(log [γ/100 g.])*			
10	1	50	374	3.12	3.08	2.61	3.42
10	5	64	480	3.49	3.27	2.81	3.66
10	25	72	762	4.08	3.40	2.94	3.82
100	1	86	170	2.91	2.71	2.31	3.16
100	5	52	425	3.27	3.17	2.67	3.50
100	25	60	470	3.37	3.24	2.77	3.55
1,000	1	67	130	2.75	2.60	2.31	3.08
1,000	5	49	320	3.19	3.08	2.66	3.40
1,000	25	77	504	3.58	3.23	2.74	3.55
Standard deviation of a calf		25	84	0.07	0.06	0.07	0.10

[a] Intake per pound live weight per day.
[b] Values represent four calves per treatment group.
[c] Values represent two calves per treatment group.

log γ/100 g. per log unit intake of tocopherol; heart, 0.35 ± 0.03 log γ/100 g.; trapezius muscle, 0.29 ± 0.03 log γ/100 g.; and perinephric fat, 0.30 ± 0.05 log γ/100 g. In the case of heart, trapezius muscle, and perinephric fat there was slight, but statistically significant negative curvature, in these relationships. However, simple correlations between log tissue concentration and log intake ranged between 0.96 and 1.00 for these particular tissues; therefore, curvature was disregarded for the purposes of this study. Expressing tissue concentration on a per gram of lipid basis did not change the corresponding relationships between tissue concentration and intake.

Vitamin A, across all levels of tocopherol intake, resulted in decreased tissue concentrations of tocopherol. These decreases per log unit intake of vitamin A were 110 ± 17 γ tocopherol per 100 ml. plasma and 0.20 ± 0.02 log γ per 100 g. for liver, 0.14 ± 0.02 for heart, 0.11 ± 0.02 for trapezius muscle, and 0.14 ± 0.03 for perinephric fat. These decreases were not consistent for each log unit intake of vitamin A, since the decrease in tissue tocopherol concentration was greater between the 10 and 100 γ intakes of vitamin A than between the 100 and 1,000 γ intakes of vitamin A. This was substantiated by the presence of significant ($P < 0.05$) departure from linearity (quadratic curvature) in the regressions of log tocopherol concentration on log vitamin A intake. The same general trends as noted above were found when the tissue concentration of tocopherol was expressed on a lipid basis. Vitamin A intake did not influence the tissue tocopherol

concentration equally across all levels of tocopherol intake, but the decrease in tocopherol tissue concentration with vitamin A intake was for heart, trapezius muscle, and perinephric fat greater at the lowest tocopherol intake of 1 mg. than at the highest. This was substantiated by statistically significant coefficients of the cross-product terms of intake, particularly, the linear tocopherol intake times the linear vitamin A intake interaction.

During vitamin A depletion, plasma tocopherol (expressed in logarithms in the regressions of Table 5) decreased very rapidly to levels of approximately

TABLE 5

Effect of tocopherol–vitamin A supplementation on plasma tocopherol concentration change and terminal plasma and liver tocopherol concentration during vitamin A depletion

Vitamin A intake[a]	Tocopherol intake[a]	Regression of log plasma tocopherol concentration on weeks of vitamin A depletion [b, c]	Terminal plasma tocopherol [b, d]	Terminal liver tocopherol [b, d]
(γ)	*(mg.)*		*(γ/100 ml.)*	*(log [γ/100 g.])*
10	1	$\hat{Y} = 2.58 - 0.115\ X \pm 0.17$, (83, 5)[e]	162	2.55
10	5	$\hat{Y} = 2.62 - 0.108\ X \pm 0.16$, (96, 5)	155	2.58
10	25	$\hat{Y} = 2.68 - 0.108\ X \pm 0.24$, (88, 5)	187	2.63
100	1	$\hat{Y} = 2.32 - 0.120\ X + 0.007\ X^2 \pm 0.52$, (50, 16)	98	2.33
100	5	$\hat{Y} = 2.67 - 0.142\ X + 0.007\ X^2 \pm 0.17$, (93, 13)	97	2.34
100	25	$\hat{Y} = 2.70 - 0.135\ X + 0.007\ X^2 \pm 0.23$, (88, 15)	96	2.37
1,000	1	$\hat{Y} = 1.87 - 0.016\ X + 0.001\ X^2 \pm 0.57$, (54, 29)	107	2.25
1,000	5	$\hat{Y} = 2.32 - 0.051\ X + 0.001\ X^2 \pm 0.73$, (52, 35)	111	2.33
1,000	25	$\hat{Y} = 2.27 - 0.038\ X + 0.001\ X^2 \pm 0.74$, (49, 41)	113	2.33
Standard deviation of a calf			32	0.05

[a] Intake per pound live weight per day.
[b] Values represent two calves per treatment group.
[c] Regression of log plasma tocopherol in γ/100 ml., Y, on weeks, X, during vitamin A depletion until each calf's blood plasma vitamin A decreased to $\leqslant 4.0$ γ/100 ml.
[d] Terminal value for each calf when blood plasma vitamin A had decreased to $\leqslant 4.0$ γ/100 ml. for two consecutive seven-day periods.
[e] First value in parentheses = per cent of variation about log plasma tocopherol concentration accounted for by regression; second value = number of weeks included in regression.

100-150 γ/100 ml. and then, in the case of calves receiving previously the 100 and 1,000 γ vitamin A intakes, increased slightly (Table 5). Separate analyses of the linear and quadratic polynomial coefficients from which the regressions in Table 5 were derived, indicated some effect of previous vitamin A intake, i.e., the calves fed the higher vitamin A intakes, which resulted in greater vitamin A depletion times, had rates of decrease of plasma tocopherol which were less than the calves fed the lower vitamin A intakes. However, these differences were slight and, with the data at hand, impossible to differentiate between the effect of previous vitamin A intake and length of the vitamin A depletion period. The terminal plasma and liver tocopherol values (Table 5) were directly related to the previous vitamin A intake or to the length of vitamin A depletion. Also, liver tocopherol was influenced by the previous tocopherol intake by the amount of $+0.05 \pm 0.02$ log γ tocopherol per 100 g. of liver for each log unit increase of previous tocopherol intake at any given vitamin A intake.

DISCUSSION

These data confirm and extend previous findings, as reviewed in the introduction, that dietary tocopherol may increase as well as decrease the utilization of vitamin A in the dairy calf. Tocopherol caused increases in utilization of vitamin A at the highest intake of the latter, decreases at the lowest intake of vitamin A, and little change at the middle intake of this vitamin. Therefore, these data suggested that for a given intake of vitamin A, there exists a level of intake or a range of intakes of tocopherol which provides for maximum utilization of vitamin A. It is possible that for maximum utilization of vitamin A, a common ratio of tocopherol intake to vitamin A intake exists. However, the experiment reported herein unfortunately did not provide a range of intakes of both vitamins which would allow testing of such a hypothesis.

Whether or not tocopherol acts on vitamin A only in the alimentary tract or in the body proper is controversial (17, 21, 22). Evidence from this experiment indicated, but not unequivocally, that the majority of the activity occurs in the alimentary tract as shown by a change in vitamin A tissue (plasma and liver) concentrations at the end of tocopherol–vitamin A supplementation, and, also, that some of its activity occurs within the body proper, as evidenced by slight changes in the rate of decrease of plasma vitamin A due to previous tocopherol supplementation during vitamin A depletion. It is recognized, in the latter, that the differences were not statistically significant; however, these rates of change somewhat paralleled those for vitamin A depletion time, as well as plasma and liver concentrations at the completion of tocopherol–vitamin A supplementation. Had the free form of α-tocopherol been fed in contrast to α-tocopheryl acetate (which does not exhibit antioxidant activity until hydrolyzed into the phenolic form, presumably at the time it passes into the intestinal tract), possibly larger differences in storage of vitamin A at the end of tocopherol–vitamin A supplementation in relation to the slight change in plasma vitamin A values during vitamin A depletion might have occurred (5, 18, 22). Previous work (28), in which several antioxidants were fed to calves with artificially dehydrated alfalfa as a source of carotene and in which one-half of the calves from each antioxidant group were depleted of vitamin A after completion of carotene-antioxidant supplementation, would tend to support the hypothesis that the major site of antioxidant activity is the alimentary tract.

The decrease in tissue tocopherol concentration due to increasing vitamin A intake was of interest, for it has become increasingly evident, since the excellent review of Moore (21), that the intake of one fat-soluble vitamin, especially at high intakes of one in relation to a second, influences the biological activity of the second. Recently, Irving (13) observed that the administration of large amounts of vitamin A from a fish liver oil concentrate to rats diminished the protective action of α-tocopherol upon tooth color, incisal enamel organ, and dialuric acid hemolysis test. Of further interest is the finding (32) that the response of calves to carotene intake was different in the presence of dietary vitamin D than in its absence. High intakes of carotene or vitamin A have been observed to be rachitogenic for rats (9, 33) and have been interpreted to be

the cause of rickets or ricket-like conditions observed in sheep on rapidly growing pasture, especially during the winter months when ultraviolet radiation was low. Thus, the data herein and the above-mentioned experiments point to the necessity of recognizing the interrelationships among the fat-soluble vitamins, which is especially important when evaluating the adequacy of a ration with respect to a particular fat-soluble vitamin.

While this experiment indicated some of the interrelationships between tocopherol and vitamin A, these may not hold for other forms or sources of either vitamin. There is also a need for further work to quantitatively establish levels of intake or ratios of intake for both vitamins to provide optimum utilization of either or both vitamins.

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EFFECTS OF TWINNING ON REPRODUCTIVE EFFICIENCY IN A HOLSTEIN-FRIESIAN HERD [1, 2]

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SUMMARY

Out of 7,387 calvings in a Holstein-Friesian herd covering a 30-yr. period, 338, or 4.58%, were multiple births. Two of these were triplets. The twin combinations were 108 ♂ ♂ : 154 ♂ ♀ : 74 ♀ ♀. An estimated 13.68 ± 0.90% of the like-sexed twins were monozygous (maximum likelihood method). Twin calves were 55% males, as compared with 51.65% for the herd. Twinning rate was 1.3% for heifers and 4.4% at second calving ($P < 0.001$). Rates increased to 7.1% at the tenth calving (not significant). Mortality among twin calves was high by parturition time, averaging 41% of the males and 35% of the females. Twin cows reproduced as well as their singly born herd mates. Cows having twins before the fourth calving reproduced fewer times subsequently. Incidence of relative infertility was significantly greater after twinning, though average services per conception and calving intervals were not consistently different. Twinning is undesirable in dairy cattle because of increased infertility and a reduced number of female progeny from cows which have twins.

Multiple births in some species are a desirable and natural trait and an indication of high reproductive capacity. In a uniparous species, such as the cow, multiple births are comparatively rare, though there is considerable variation between breeds (19, 23) and families (18, 21) and daughters of sires (5, 18, 21) within a breed.

Several factors point to the economic undesirability of twinning in cattle. This characteristic shows no consistent relationship with yield (4, 19); therefore, economic advantage could be gained only through a greater number of progeny for subsequent generations. Pfau *et al.* (21) have indicated that fewer female progeny are available because of increased losses at birth, high fertility rates among living females from twin births of unliked-sex, and lowered vitality of other twin calves. The indications are that cows producing twins are more subject to post-partum difficulties (3, 6, 7, 20), which endanger the life of the cow and impair subsequent reproduction (3, 4, 21).

This study was conducted to evaluate the influence of twinning on reproductive performance in a large herd of Holstein-Friesian cows.

SOURCE OF DATA

The data used for this study were from the herd records of Carnation Milk Farms, Carnation, Washington, and they cover a period of 30 yr. Detailed ac-

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counts of the records available, and methods of handling the data, have been described before (6, 9, 11, 20).

tatistical methods were restricted to Chi-square and confidence limits, as described by Snedecor (22).

RESULTS AND DISCUSSION

Out of 7,387 calvings, an estimated 338, or 4.58%, were multiple births. Two of these were triplets. Each set consisted of two males and a female. One set aborted at 161 days and the other at 187 days. Among the 336 twin births, 311 actually were observed. Twenty-five twinnings were estimated for the 245 abortions where the number of fetuses and sexes were unknown (20). This estimate was based on the statistic that 10.4% of the abortions of known sex were twins. Among 29 abortions of twins of known sex, 41.4% were males, 17.2% were females, and 41.4 were unliked-sexed. These distributions were used to assign the 40 twin abortions of unknown sex to the sex combinations. The combinations were 108 ♂ ♂ : 154 ♂ ♀ : 74 ♀ ♀.

Holstein twinning rates. Meadows and Lush (19) recently summarized 18,736 Holstein calvings which seemed to be from herd records. The twinning rate was 3.08%. This summary contains 2,910 births reported by Knott (17) for the Carnation herd. Removing the data of Knott (17), and adding in the present study, brings the total to 23,213 births, with an average twinning rate of 3.57%. The 4.58% rate for the Carnation herd is higher than generally observed for this breed. However, more abortions are likely included than is generally the case; excluding the 25 sets estimated for 245 abortions where the number of fetus and sexes were unknown, reduces the rate to 4.24%. Weber (23) has tabulated multiple-birth rates for European cattle. The Simmentaler had the highest rates. The sizable breed differences (19, 23) indicate that twinning tendency is a heritable trait; therefore, one can expect rather wide variations between herds within a breed.

Monozygous twins. Using the maximum likelihood method of Bonnier (1), an estimated 13.68 ± 0.90% of the like-sexed twins were monozygous. The value was 7.4% for all twins and 0.3% for all births. The occurrence of monozygous twins has been variously estimated. When expressed in terms of total twins, the following have been observed: *a*) Bonnier; (1) : 4.4%; *b*) Johansson (15) : 6.0%; *c*) Meadows and Lush (19) : 4.7%, and *d*) Weber (23) : 1.7%.

Sex ratios. Among all births of known sex, 51.65% were males, a value very near the 51.52% reported by Johansson (15). Sex ratios may be quite different, depending on whether registrations or herd records are used. In this study, 50.92% were males out of 5,524 living single calves of known sex. Similarly, the single stillbirths were 56.76% males ($P < 0.25$) and the single abortions were 59.39% males ($P < 0.05$). The average for all single births was 51.55% males. Since sex was not always recorded (245 cases out of 668 total abortions), the estimate of 51.55% for single births probably is too low. Erb and Holtz (8) estimated embryonic loss at 20.6% for this herd. Chapman *et al.* (2) reported a highly significant change in fetal sex ratios, which apparently was due to

higher mortality among males after the 5–10 cm. stage was reached. Out of 126 individuals in the 5–10 cm. stage, 66% were males (2).

Among twins, in the present study, 55% were males ($P < 0.25$) as compared with the 51.55% for all single births. Twins living at birth were 52.6% males compared with 55.6% of the calves dead at birth and 61.6% of those aborted. These ratios are not significant when similarly compared with single living, stillborn, and abortive births.

Age of dam. There were 268 sets of twins from cows which had their first calves in the herd. Frequency of twinning by parturition number (age) of the dam for this and a similar study (23) is shown in Figure 1. Significantly fewer

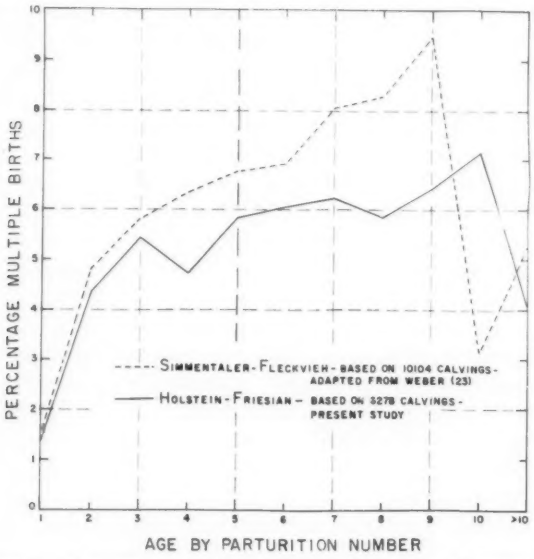
PERCENTAGE MULTIPLE BIRTHS
AGE BY PARTURITION NUMBER
SIMMENTALER-FLECKVIEH - BASED ON 10104 CALVINGS - ADAPTED FROM WEBER (23)
HOLSTEIN-FRIESIAN - BASED ON 3278 CALVINGS - PRESENT STUDY

FIG. 1. Twinning rates among cows of different ages (age determined by parturition number).

($P < 0.001$) heifers than older cows produced twins. The greatest increase was between the first (1.3%) and second (4.4%) parturitions of Holsteins. These rates are very similar to the rates among Simmentalers (23) for the first two parturitions, but the latter continued to show increases in twinning rates through the ninth calving. Cows of both breeds showed evidence of declining rates after 10 yr. of age. Others (14, 16, 21) have noted changes in twinning frequency with age.

Survival of twins. Mortality by the time of birth was 38% (17% stillborn and 21% aborted) for twin calves. Male losses were higher (41%) than female losses (35%). Figure 2 shows that 87.5% of singly born calves were living at birth, as compared with 73.3% of like-sexed female twins and 56.0% of the like-sexed male twins. Among unlike-sexed twins, more males (63.4%) were living at

birth than females (58.3%). Pfau *et al.* (21) found a slightly higher mortality rate among males, and the average for both sexes was 22.7%. Weber (23) similarly observed an average of 28.8%.

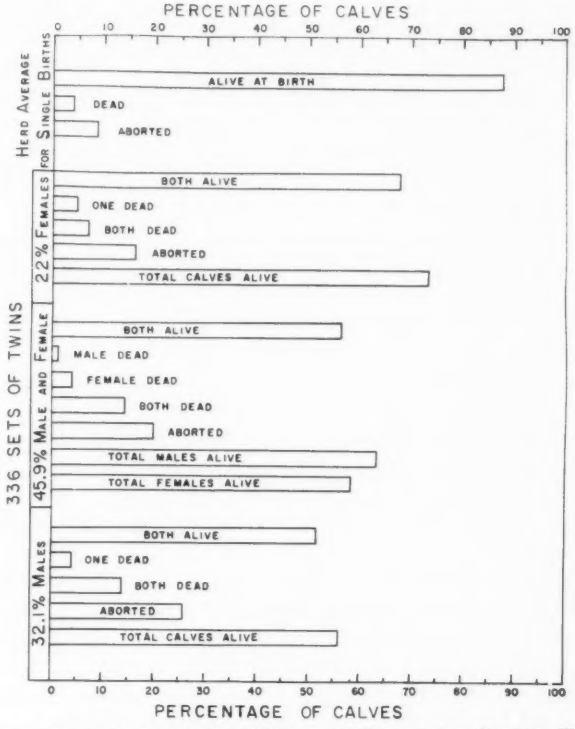

FIG. 2. Comparisons of abortions, stillbirths, and living calves between like- and unlike-sexed twins.

During the study, 198 females from twin births were alive, but 91 of these were born with males. Assuming that 9% (13) of the females born with males were fertile, the total available at birth for breeding purposes could have been no more than 115 females. If the 338 multiple births had been single births, 146 living females should have been available (20).

During the study, service records were available on only two females born with males. Each failed to calve. It can be reasonably assumed that others were not raised to breeding age. Of the remaining 107, 13 were too young for breeding when the study ended. Of the remaining 94, 82 were bred and all but five conceived. Thus, 13% of those alive at birth were not available for breeding. The average for singly-born females was approximately 15%. These percentages represent losses from death after birth and those sold for dairy purposes before breeding.

Thirty (32%) of the female twins were sold for dairy purposes as bred heifers. An estimated 29% of singly born females were sold as bred heifers. Thus, there is little evidence that death losses or selection pressure for female twin calves (like-sexed) living at birth were different from those of singly born females. Therefore, a comparison of number of calvings for those which calved one or more times before disposal should indicate if twin females were less likely to remain in the herd. The herd average was 3.9 calvings for 1,602 cows having first and succeeding calvings in the herd (7); the 47 female twin cows averaged 4.1 calvings. Thirteen per cent of twin cows disposed of during the study were relatively infertile; the herd average was 16% (20). The evidence is that twin cows reproduce as well as their singly born herd-mates. Pfau *et al.* (21) have made a similar observation.

Influence on subsequent reproduction. Pfau *et al.* (21) observed that after bearing twins, cows had longer calving intervals and required more services for conception. Cloninger and Thoele (4) found that 110 cows required 2.0 services for conception before twinning and 2.6 immediately after. Previous summaries of the present data showed that abnormal calvings (abortions, twins, stillborns, or retained placentae) were not a serious cause of faulty subsequent reproduction, provided the cows remained free of after-calving disorders (7). However, reproductive disorders were more frequent after retained placentae (7) and other types of abnormal calvings (6, 7). Estrus after conception (11) and twins (3, 12) occurs more frequently immediately following cystic ovaries. The placenta was retained three to five times more often following twin births (7, 10, 20).

Evaluation of the effects of twinning on subsequent fertility is difficult, because twinning occurs more frequently among older cows and because of the association with post-parturient disorders. One approach (Table 1) was to compare the average number of calvings after initial twinning with the average for the remaining cows. As Table 1 shows, calving expectancy was reduced for cows twinning initially at one of the first three calvings. The percentage of cows leaving the herd averaged $31 \pm 3.7\%$ for twinning cows and $20 \pm 0.5\%$ for herd-mates ($P < 0.01$). This latter comparison is identical to a similar comparison made for retained placentae cases (7).

The usual criteria for measuring reproductive efficiency are shown in Table 2, for normal and abnormal cows, following birth of single or twin calves, with and without retained placenta. Cases of aborted twins were not included. Cows with no known pathological condition other than cystic tendency (normals) following birth of twins required slightly more services for conception when the placenta was not retained, and slightly fewer when the placenta was retained, than cows after single births. After twinning, the average calving interval was 2–3 wk. longer. Similar comparisons for abnormal cows indicate considerably less reproductive trouble after twins than for singles, when retained placenta was not involved. This result may have been due to closer observation after twinning, resulting in diagnosis of a higher proportion of relatively minor disorders.

TABLE 1

Evidence for greater infertility subsequent to twinning as based on calving expectancy

Calving period	Herd total[a]			Twinning groups[a]		
	Cows calving[b]	Average/ cow afterward	Per cent not calving	Cows calving	Average/ cow afterward	Per cent not calving
1	915	3.8	19	11	3.1	27
2	718	3.6	15	33	2.7	30
3	603	3.5	14	28	1.9	32
4	516	3.0	16	19	3.0	21
5	426	2.6	23	22	2.3	27
6	336	2.1	23	10	2.2	10
7	256	2.0	21	13	1.5	38
8	204	1.5	35	7	1.1	57
9	129	1.3	35	7	1.4	29
<9	171	0.8	49	7	1.0	57
Total	4,274	3.0	20	157	2.2	31

[a] Does not include cows sold for dairy purposes and those in the herd before and after the study.

[b] Cows having twins were excluded.

The reproductive life of cows was lessened after twinning (Table 1), primarily because a proportionately greater number had post-parturient disorders (Table 2) as compared with single births. The common measures of reproductive efficiency failed to show any marked differences between the groups compared (Table 2). The primary difference was that more cows were disposed of without conceiving, particularly if twinning occurred during one of the first three calving periods (Table 1).

TABLE 2

Influence of twinning on subsequent reproductive efficiency of the dams

Group	No. of cases	Yr. of age	No. conceived	S/C	Total S/C	Calving interval	Average days in herd and not conceived[a]
No pathogenic condition[s] observed after the birth[b]							
Living single—no R.P.[c]	3,858	5.9	3,161	1.9	2.2	402	191
Living single—R.P.	204	7.9	141	2.3	2.7	424	216
Twins—no R.P.	98	7.1	74	2.2	2.4	415	92
Twins—R.P.	58	7.7	39	2.1	2.3	447	125
Pathogenic condition[s] observed after the birth[b]							
Living single—no R.P.	1,890	6.0	1,554	3.3	4.6	495	542
Living single—R.P.	225	8.1	162	3.9	5.5	511	516
Twins—no R.P.	50	6.9	44	2.5	2.8	473	553
Twins—R.P.	61	7.7	41	3.6	5.6	516	575
Twins—total	267	7.3	198	2.5	3.1	454[d]	282
Herd average	9,994	5.9[e]	8,409	2.5	3.0	442[f]	200

[a] Refers to cows not again conceiving—includes death, culling, and infertility.

[b] Refers to all genital abnormalities except cystic tendency.

[c] R.P. = retained placenta.

[d] 183 cows—15 were sold pregnant.

[e] Average includes age at calving, age at first conception for heifers, and age at disposal for those not conceiving. Age at calving for 7,387 total calvings was 6.1 yr.

[f] 7,387 calvings.

Twinning in dairy cattle is undesirable, because the characteristic is associated with *a*) greater infertility, *b*) fewer female progeny for subsequent generations, *c*) a 3- to 5-fold increase in retained placenta cases (7, 20), and *d*) increased post-parturient disorders. Subsequent study has shown that twinning is transmitted like a heritable trait (5). Though retained placenta is closely associated with twinning (7, 20, 21), the indications are that retained placenta is transmitted in an additively genetic manner (10).

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FACTORS AFFECTING THE FERTILITY OF FROZEN BOVINE SPERMATOZOA[1]

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SUMMARY

Three separate studies were conducted in an attempt to determine the most suitable extender, the spermatozoan concentration necessary, and the method of filling and sealing glass ampules, to obtain optimal nonreturn rates with frozen bovine spermatozoa.

In the first experiment, a total of 934 first-service cows were bred with semen extended and frozen in yolk-citrate extender, 945 cows with milk, 928 cows with yolk-glucose-glycine, and 902 cows with skimmilk. The 75-day nonreturn rates were 64.7, 66.2, 41.3, and 65.3%, respectively. Yolk-citrate, milk, and skimmilk, on the basis of this experiment, were acceptable semen extenders for frozen semen, whereas yolk-glucose-glycine was not suitable.

For the second experiment, 469 first-service cows were bred with frozen semen containing 10 million live spermatoza per insemination at time of initial dilution, 479 cows with frozen semen containing 20 million spermatozoa, and 472 cows with semen containing 30 million spermatozoa per insemination. The 75-day nonreturn rates for the respective levels were 63.1, 68.9, and 72.7%. The difference between the 10- and 30- million level was highly significant ($P<0.01$). The other differences were not significant.

In the third experiment, 294 first-service cows were inseminated with frozen semen for which the glass ampule had been gravity-filled–tip-sealed, 275 cows inseminated with frozen semen in ampules that had been gravity-filled–cut, pull-sealed; 313 cows inseminated with frozen semen in ampules that had been forced-filled–tip-sealed, and 254 cows inseminated with frozen semen in ampules that had been force-filled–cut, pull-sealed. The 75-day nonreturn rates for the respective methods of filling and sealing were 69.0, 73.8, 63.9, and 70.5%. None of the differences was significant. However, a significant difference existed between tip-sealed and pull-sealed, irrespective of method of filling. The difference between gravity-filled and force-filled, irrespective of method of sealing, was not significant.

A considerable amount of information is now available concerning the freezing and low-temperature storage of bovine spermatozoa. However, many phases have been investigated only to a limited extent. Optimal fertility results depend upon a further knowledge of various factors affecting frozen bovine spermatozoa. Three significant factors are semen extenders, spermatozoa concentrations, and the method employed in filling and sealing the ampules.

Yolk-citrate was the extender used by (1, 9, 11, 13, 14, 18) in the early British work on frozen semen. Dunn and Hafs (3) reported that the difference was not significant when comparing the mean motility percentage and revival rates of semen samples frozen in milk and yolk-citrate. O'Dell and Almquist (12) observed that homogenized milk and skimmilk extenders gave spermatozoa revival rates equal to the yolk-citrate extender. Bruce (2) reported no difference in a fertility trial comparing yolk-citrate with skimmilk extender. Rakes and Stallcup (15) compared the average per cent motility of semen samples extended in yolk-

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citrate extender and in yolk-glycine extenders containing various percentages of glycine, that were frozen and stored for seven days. The results reported were very similar for each of the variables checked.

Several British workers (14, 18, 19) reported the usage of very low semen extension rates in their initial frozen semen studies. Rowson (16) reported a limited number of cows inseminated with frozen semen extended at various rates. Twelve cows were inseminated with frozen semen extended 1:10, and 11 cows each with frozen semen extended 1:20, 1:30, and 1:40. Conception rates for the respective groups were 50.0, 63.6, 81.8, and 63.6%. American workers initially used higher extension rates in their work on frozen semen. The laboratory results of Miller and VanDemark (10) indicated that sperm survival following freezing was not affected by dilutions to 10, 30, and 90 million sperm per milliliter. Dunn *et al.* (4) reported that the 60- to 90-day nonreturn rates for inseminations made with frozen semen containing 5, 12, and 30 million spermatozoa per insemination were 52.6, 59.0, and 61.3%, respectively. Bruce (2) conducted a relatively large scale trial to check the effect of semen extension rates of 1:20 and 1:50. The 1:20 extension rate resulted in a 16-wk. nonreturn rate of 55.2% on 1,227 first services, and 52.9% on 1,235 first services for the 1:50 extension rate. The difference was not significant.

Up to the present time, no information has been reported concerning the possible effect on the semen that the method of filling and sealing may have on the subsequent fertility rate. For mass preparation of frozen semen, fully automatic filling and sealing machines will become a necessity. Some such machines are equipped with gravity-flow filling devices and others with a forced-ejection apparatus. In combination with the filling mechanism, some automatic machines seal the glass ampules either by a tip seal or by a pull seal, that require different intensities of heat.

To more fully study these effects on the fertility of frozen bovine spermatozoa by different extenders, sperm concentrations, and method of filling and sealing the glass ampules, three separate experiments were conducted.

EXPERIMENT I

This study was conducted to determine which extender material would be most suitable for freezing bovine spermatozoa, based on nonreturn rates. Four semen extenders were employed in the experiment, as a result of extensive laboratory studies.

Experimental procedure. Semen was collected from six Holstein sires, one at each of six different artificial breeding organizations. Selection of the sires was done by the management of the respective organizations and based only on demand for the sire. It was necessary to obtain two ejaculates of semen and, occasionally, a third ejaculate, in order to provide a sufficient amount of semen. The semen was maintained at 30° C. until all ejaculates were completed. The pooled ejaculates were thoroughly mixed and the split, mixed ejaculate technique employed. The temperature of semen and extenders at time of semen extension was 30° C.

The composition and final percentage of the constituents in each of the four extenders are presented (Table 1). Immediately after semen collection, sufficient

TABLE 1

Composition and final percentage of the constituents for each extender used

Yolk-citrate[a, b]		Yolk-glucose–glycine[a, c, d]	
Composition	(%)	Composition	(%)
Fresh egg yolk	23.25	Fresh egg yolk	23.25
Trisodium citrate (dihydrate)	2.02	Glucose	1.04
Glycerol	7.00	Glycine	2.05
		Glycerol	7.00
Heated, pasteurized, homogenized whole milk[a]		Heated, pasteurized skimmilk[a]	
Composition	(%)	Composition	(%)
Milk	90.00	Skimmilk	90.00
Glycerol	10.00	Glycerol	10.00

[a] Penicillin (potassium crystalline G) and dihydrostreptomycin sulfate were added to each extender at a rate of 1,000 units and 1,000 μg. per milliliter, respectively (5).

[b] Yolk-citrate extended on a 100-ml. basis, prior to addition of glycerol, consisted of 75 ml. of a 2.9% trisodium citrate (dihydrate) solution and 25 ml. of fresh egg yolk.

[c] Yolk-glucose–glucose-glycine extender on a 100-ml. basis, prior to addition of glycerol, consists of 20 ml. of a 5.6% glucose solution, 55 ml. of a 4.0% glycine solution, and 25 ml. of fresh egg yolk.

[d] pH adjusted to 6.8 with sodium hydroxide.

semen was added to 10 ml. of each extender. This small amount of extended semen was cooled to 5° C., which required about 3.5 hr. The glycerolated extender was then added to the extended semen in four steps at 10-min. intervals, in progressively larger quantities to yield an extension rate of 1:40 on final extension of the glycerolated extender. The extended, glycerolated semen was equilibrated for 12 hr. at 5° C. prior to the filling and sealing (7). Glass ampules were utilized.

Freezings were conducted according to the method described by Graham and Marion (8). The rate of freezing for the semen extended in yolk-citrate and yolk-glucose-glycine was 3° C. per minute from 5 to −15° C. and 5° C. per minute from −15 to −79° C. (5, 6). The semen extended in milk and skimmilk was frozen at a rate of 2° C. per minute from 5 to −30° C., and 5° C. per minute from −30 to −79° C. (5, 6). Following freezing, the semen was stored at −79° C., using dry ice and methanol.

Field trials were conducted in three parts, representing three different semen collection periods for the six different bulls. Thirty-three technicians participated in the trial. Twelve ampules of frozen semen, three of each extender, were sent each week to the technicians for five consecutive weeks. The technicians selected the semen vials at random from their dry ice field kits and thawed the semen in a 5° C. water bath, using the semen immediately following thawing, on first-service cows. An IBM method was utilized for the reporting of inseminations and for summarizing the data for the determination of per cent nonreturns.

Motility estimations of the semen were made before freezing, after freezing, and each week during the course of the trial, to obtain additional information. The motility estimations were based on percentage of progressively motile spermatozoa observed microscopically.

Results and discussion. A summary of the nonreturn rates for the different extender materials used is presented (Table 2). Nonreturn rates were based on

TABLE 2

Over-all nonreturn rate for the various types of extenders

Variable	First services	N.R.	N.R. 75-days
	(No.)	*(No.)*	*(%)*
Yolk-citrate	934	604	64.7
Milk	945	626	66.2
Yolk-glucose-glycine	928	383	41.3
Skimmilk	902	589	65.3
Total	3,709	2,202	59.4

75-day nonreturns that should be comparable to 60- to 90-day nonreturns, which places every animal on an equal basis irrespective of the time of the month it was bred. The difference of 1.5, 0.9, and 0.6% between milk and skimmilk, milk and yolk-citrate, and skimmilk and yolk-citrate, respectively, based on a 75-day nonreturn rate, was not significant as analyzed by Chi-square (17). However, there was a highly significant difference ($P < 0.01$) between these three extenders and the yolk-glucose-glycine extender.

The nonreturn rate for individual bulls and extenders used is presented in Table 3. Two sires had the highest nonreturn rate when their semen was frozen in yolk-citrate, two in milk, and two in skimmilk. The results of Chi-square

TABLE 3

Nonreturn rate for individual bulls and extenders used

Bull number	First services	N.R.	N.R. 75-days	First services	N.R.	N.R. 75-days
	(No.)	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*
	Yolk-citrate				Milk	
H–1	158	100	63.3	165	114	69.1
H–2	159	114	71.7	155	98	63.2
H–3	171	89	52.0	172	86	50.0
H–4	161	107	66.5	164	129	78.7
H–5	128	83	64.8	127	86	67.7
H–6	157	111	70.7	162	113	69.8
Total	934	604	64.7	945	626	66.2
	Yolk-glucose-glycine				Skimmilk	
H–1	158	83	52.5	151	112	74.2
H–2	156	65	41.7	154	106	68.8
H–3	166	48	28.9	167	89	53.3
H–4	157	66	42.0	154	110	71.4
H–5	128	52	40.6	120	68	56.7
H–6	163	69	42.3	156	104	66.7
Total	928	383	41.3	902	589	65.3

analyses show that the differences in 75-day nonreturns between yolk-citrate, milk, or skimmilk, and yolk-glucose-glycine extender were highly significant for all bulls, except the difference between yolk-citrate and yolk-glucose-glycine for H-1, which only approached significance, and between skimmilk and yolk-glucose-glycine for H-5, which was significant at the 5% level. Significant differences existed between yolk-citrate and milk for H-4, and yolk-citrate and skimmilk for H-1. All other comparisons were not significant.

The data demonstrate that semen from various bulls responds differently when extended in different extender materials on the basis of nonreturn rate. Yolk-glucose-glycine extender can not be considered as a suitable extender for semen to be frozen. Yolk-citrate, milk, and skimmilk can be considered suitable extenders; however, none of these three can be considered as the most suitable for all bulls.

The similar nonreturn rates obtained for yolk-citrate and skimmilk agree with the findings of Bruce (2), who compared reconstituted skimmilk powder and yolk-citrate on the basis of fertility results.

The nonreturn rates for the extenders used, by storage time intervals, are presented in Table 4. The nonreturn rate for the 1st wk. of storage served as the control and was compared with the nonreturn rate of each of the remaining weeks of storage by extenders used. Also, the over-all nonreturn rate for the first week of storage was also compared with the over-all nonreturn rate for each of the remaining weeks of storage. In no instances were the differences significant.

Correlation and regression were determined for per cent progressively motile spermatozoa on the nonreturn rate, the length of storage by weeks on the nonreturn rate, and length of storage by weeks on per cent progressively motile spermatozoa. The basis for the over-all correlation and regressions were each treatment, each semen collection period, each bull, and each week of storage. A correlation between per cent progressively motile spermatozoa on the nonreturn rate was .251 ($P < 0.01$), with a regression of +.588% in nonreturn rate for each per cent increase in progressively motile spermatozoa. A negative and insignificant correlation existed between length of storage by weeks and the nonreturn rate. The regression in the case was a decrease of .418% in nonreturn rate for each additional week of storage. A negative, highly significant correlation existed between length of storage by weeks and per cent progressively motile spermatozoa. The regression was a decrease of 1.643% progressively motile spermatozoa for each added week of storage. Even though two of the correlations were highly significant ($P < 0.01$), they are not great enough to be useful in the prediction of a factor on the basis of the other related factors.

EXPERIMENT II

This study was carried out to determine the extent that bovine semen can be extended and still provide optimum fertility results when frozen.

Experimental procedure. Semen was collected from seven Holstein bulls located at five different artificial breeding organizations. Yolk-citrate of the composition described in Experiment I was the extender used. All processing pro-

TABLE 4

Nonreturn rate for storage-time interval and extenders used

Type of extender	First services	N.R.	N.R. 75-days
1-wk. storage	*(No.)*	*(No.)*	*(%)*
Yolk-citrate	193	118	61.1
Milk	196	141	71.9
Yolk-glucose-glycine	187	80	42.8
Skimmilk	195	134	68.7
Total	771	473	61.3
2-wk. storage			
Yolk-citrate	205	137	66.8
Milk	205	133	64.9
Yolk-glucose-glycine	204	81	39.7
Skimmilk	200	123	61.5
Total	814	474	58.2
3-wk. storage			
Yolk-citrate	203	125	61.6
Milk	194	122	62.9
Yolk-glucose-glycine	192	83	43.2
Skimmilk	186	129	69.4
Total	775	459	59.2
4-wk. storage			
Yolk-citrate	202	141	69.8
Milk	197	132	67.0
Yolk-glucose-glycine	202	82	40.6
Skimmilk	196	122	62.2
Total	797	477	59.8
5-wk. storage			
Yolk-citrate	131	83	63.3
Milk	153	98	64.1
Yolk-glucose-glycine	143	57	39.9
Skimmilk	125	81	64.8
Total	552	319	57.8

cedures were the same as for Experiment I, except for the semen extension rate. For this experiment, the extension rate in terms of final extension was 10, 20, and 30 million live spermatozoa per milliliter at the time of initial dilution. If field technicians of a participating artificial breeding organization were routinely using less than 1 ml. of extended semen per insemination, the number of spermatozoa for each of the three levels was calculated on a per-insemination basis. Two ampules of each spermatozoan concentration were sent to 42 field technicians each week for 7 wk.

Results and discussion. A summary of the nonreturn rates for the three spermatozoan concentrations is presented in Table 4. The difference of 5.8% in nonreturn rate between 10 million and 20 million live spermatozoa per milliliter of final extended semen, determined at the time of initial semen extension, approaches significance at the 5% level on the basis of Chi-square analysis. The difference of 9.6% in nonreturn between 10 million and 30 million live spermatozoa per milliliter was highly significant ($P < 0.01$). A significant difference did not exist between 20 million and 30 million spermatozoa per milliliter. These

TABLE 5

Over-all nonreturn rate for the various levels of live spermatozoa per milliliter of final extended semen

Level of live spermatozoa per milliliter of final extended semen[a]	First services	N.R.	N.R. 75-days
(millions)	*(No.)*	*(No.)*	*(%)*
10	469	269	63.1
20	479	330	68.9
30	472	343	72.7
Total	1,420	969	68.2

[a] Approximate number of live spermatozoa per milliliter of final extended semen determined at time of initial extension.

data indicate that a spermatozoa concentration of at least 20 million live spermatozoa per milliliter of final extended semen, at the time of initial semen extension, should be used when bovine semen is to be frozen, to obtain acceptable fertility results. Even though the differences between 20 million and 30 million live spermatozoa of final extended semen were not significant, there are indications that a higher spermatozoa concentration than 20 million live spermatozoa per milliliter of final extended semen may be advisable for highest nonreturn rate. This seems to be especially true for some bulls (as shown in Table 6). The analyses by Chi-square, however, indicated no significant difference between any of the spermatozoa concentrations by bull. This was due to the small number of first services per treatment by bull. In no instance were the nonreturn results higher for the 10 million live spermatozoa per milliliter level than with the 20 million live spermatozoa per milliliter level. Estimates of percentage of progressively motile spermatozoa by microscopic examination each week throughout the trial indicate that, on the average, approximately 7, 14, and 21 million live spermatozoa per insemination were used when the live sperm concentration per milliliter of final extended semen at the time of intial semen extension was 10, 20, and 30 million, respectively.

The results of this study are in agreement with the trend in the conception

TABLE 6

Nonreturn rates for the levels of live spermatozoa per milliliter of final extended semen

Bull No.	10 million[a]			20 million[a]			30 million[a]		
	First services	N.R.	N.R. 75-days	First services	N.R.	N.R. 75-days	First services	N.R.	N.R. 75-days
	(No.)	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*
H–1	74	48	64.9	81	59	72.8	76	58	76.3
H–2	76	43	56.6	71	43	60.6	65	45	69.2
H–3	69	42	60.9	72	50	69.4	69	51	73.9
H–4	66	37	56.1	69	40	58.0	71	46	64.8
H–5	63	39	61.9	59	40	67.8	60	40	66.7
H–6	59	42	71.2	64	51	79.7	66	48	72.7
H–7	62	45	72.6	63	47	74.6	65	55	84.6
Total	469	269	63.1	479	330	68.9	472	343	72.7

[a] Approximate number of live spermatozoa per milliliter of final extended semen determined at time of initial extension.

results obtained by Dunn *et al.* (4) with frozen semen containing 5, 12, and 30 mlilion spermatozoa per insemination.

EXPERIMENT III

This trial was conducted to determine the effect of two methods of filling and two methods of sealing glass ampules, based on fertility results.

Experimental procedure. Semen was collected from five Holstein sires at five different artificial breeding organizations. Semen processing was the same as explained in Experiment I, with yolk-citrate of the composition previously described being used as the extender. One-half of the semen was placed in glass ampules using a gravity-flow filling device and the other half was placed in the ampules using a forced-ejection automatic syringe. One-half of each of the two variables was tip-sealed and the other one-half sealed by a pull-sealing device. The amount of heat required by the cut-pull sealer was less than required by the tip-sealer. The ampules of semen were frozen and stored as described in Experiment I.

Ampules of each of the four variables, namely, gravity-filled–tip-sealed, gravity-filled–pull-sealed, force-filled–tip-sealed, and force-filled–pull-sealed, were sent to 36 field technicians each week for 4 wk. As in the other experiments, the technicians selected the ampules of semen at random from their dry ice field kit, thawed the frozen semen in a 5° C. water bath, and inseminated first-service cows.

Results and discussion. The results of the experiment are listed in Table 7. The differences in nonreturn rates among the four variables were not significant, except for the difference between gravity flow–cut, pull seal, and force filled–tip sealed. When the type of sealing was considered irrespective of method of filling, the difference of 5.8% was significant ($P < 0.01$). When the method of filling was considered, regardless of the type of sealing, the difference was not significant. It would appear, on this basis, that the pull seal is superior to the tip seal and that both forced and gravity filling can be considered as acceptable.

TABLE 7

Over-all nonreturn rate for the method of filling and sealing

Variable	First services	N.R.	N.R. 75-days
	(No.)	*(No.)*	*(%)*
Gravity-filled–tip-sealed	294	203	69.0
Gravity-filled–pull-sealed	275	203	73.8
Force-filled–tip-sealed	313	200	63.9
Force-filled–pull-sealed	254	179	70.5
Total			
Gravity-filled (irrespective of method of sealing)	569	406	71.4
Force-filled (irrespective of method of sealing)	567	379	66.8
Tip-sealed (irrespective of method of filling)	607	403	66.4
Cut, pull-sealed (irrespective of method of filling)	529	382	72.2

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BUFFERING CAPACITY OF BOVINE FEMALE REPRODUCTIVE TRACT FLUIDS [1]

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SUMMARY

Buffering capacities were determined for reproductive tract fluids collected from 191 cows being slaughtered at a meat-packing plant. On the average, the buffering capacity of uterine, oviduct, and follicular fluids was about 0.0225, whereas that for cervicovaginal mucus was 0.0132. Uterine fluid was more highly buffered against base than against acid. These buffering capacity values were similar to those for seminal plasma and were somewhat higher than values for blood serum.

Mucous membranes lining the cavity of bovine female reproductive organs nearly always are moist. The amount and character of these fluids vary according to location, source, and stage of estrous cycle. It seems certain that such luminal fluids of the female genitalia constitute a major part of the physicochemical environment of spermatozoa for a period of 12–24 hr. or more after insemination during estrus.

Previous studies on the composition of luminal fluids in bovine female genitalia (2) and the in vitro behavior of spermatozoa in these fluids (3) have been reported. It was found, for example (2, 3), that the pH of vaginal mucus was initially about 7.8, but after 3.5 hr. incubation at 37° C. in a Warburg flask (with KOH in the center-well) the pH rose to 9.6. On the other hand, if semen was mixed with mucus and then incubated as before, the pH dropped to about 5.3. In the case of follicular fluid, the initial pH was about 7.1, and after 3.5 hr. incubation in a Warburg flask (with KOH in the center-well) at 37° C., the pH rose to 9.3. If semen was mixed with follicular fluid before incubation, the pH remained at about 7.1. These observations suggested that buffering capacity of the luminal fluids might be of considerable importance and that there might be significant differences among the various fluids in this respect.

The following study was undertaken for the purpose of obtaining general information on the buffering capacity of cervicovaginal mucus and uterine, oviduct, and follicular fluids of cows.

MATERIALS AND METHODS

Reproductive organs were obtained from 191 cows being slaughtered by a local meat-packing plant. In most cases, the fluids were collected within 1 to 8 hr. after the cows had been killed. Mucus was collected by aspiration with a syringe after longitudinal incision of the vagina. Uterine fluid was collected by

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separating the uterine horns to the point of bifurcation, incising the broad ligament, transecting the uterus near the tubal end, and manually "stripping" the horns from the bifurcation toward the tubal end. Oviduct fluid was collected by dissecting the oviduct free of the mesosalpinx, thus straightening the tube, and stripping it either manually or with smooth forceps. Follicular fluid was obtained by aspiration with a syringe and needle. Any obviously abnormal fluids were excluded from the study.

According to Van Slyke (5), buffering capacity may be defined as the number of gram equivalents of strong acid or base required to produce a change of one unit in the pH of 1 liter of sample. Although the number of gram equivalents of acid or base required to produce a change of one unit in the pH of water increases by multiples of ten for each unit on each side of neutrality, the gross amount of acid or base remains relatively very small until the extremes are reached. Thus, it appears that Van Slyke's definition of buffering capacity has practical value when applied to physiological solutions having pH's well within the boundaries of pH 3 and pH 11 (this represents the writer's opinion).

As used in this study, buffering capacity may be calculated by means of the following formula:

$$\text{B.C.} = \frac{1}{\text{wt. of sample}} \times \text{milliliter of acid or base} \times N \times \frac{1}{\text{pH change}}$$

If, as in the case of the present study, 0.1 ml. of 0.1 N acid or base is added to the sample, the formula for buffering capacity may be reduced to:

$$\text{B.C.} = \frac{1}{(\text{wt. of sample})\ (100)\ (\text{pH change})}$$

In most cases, samples of 2.0 g. or 2.0 ml. were used, and pH was determined with a Beckman pH meter before and after the addition of 0.1 ml. of 0.1 N acid or base.

For purposes of comparison, the buffering capacity of a solution relative to water can be calculated as follows:

$$\text{Relative B.C.} = \frac{\triangle \text{ pH of sample}}{\triangle \text{ pH of an equal amount of water}} \times 100$$

This comparison can be facilitated by the following formula, which simplifies the determination of the change in pH brought about when 0.1 ml. of 0.1 N acid or base is added to an equal amount of water:

$$\text{Relative B.C.} = \frac{\triangle \text{ pH of sample}}{5 - \log \text{ of sample weight}} \times 100$$

If the relative buffering capacity turned out to be 10%, we would say that the addition of acid or base to the sample produced only 10% as much change in pH of the sample as would have occurred if the same amount of acid or base was added to an amount of water which was equal to the volume of sample.

RESULTS

The buffering capacities of cervicovaginal mucus, and uterine, oviduct, and follicular fluids are shown (Table 1). An average of 16.5 determinations were made on each fluid, with each sample representing fluids collected from an average of 8.0 cows. Statistical analysis revealed that oviduct and follicular fluids had significantly greater buffering capacities against acid than did mucus or uterine fluid. The relative buffering capacity was about 10% for mucus and uterine fluid, and 4% for oviduct and follicular fluids. It should be remembered that the lower percentage values indicate higher buffering capacity. In other words, the addition of acid to follicular fluid changes the pH only about 4% as much as would occur in an equal amount of water. Also, we could say that when acid is added to mucus or to uterine fluid, the pH would change about twice as much as it would in oviduct fluid or in follicular fluid.

TABLE 1

Average buffering capacity of bovine reproductive tract fluids against acid and base

B.C. against	Fluid from Vagina	Uterus	Oviduct	Follicle
Acid	.0136	.0139	.0221	.0243
Base	.0128	.0308	.0217	.0231
Average	.0132	.0219	.0219	.0237

The buffering capacities of reproductive tract fluids against base are also shown in Table 1. When tested by the analysis of variance, these differences were just barely significant at the 5% level of probability. However, testing by the sequential D method, as outlined by Snedecor (4), indicated that the only significant difference was that between uterine fluid and mucus. For some unknown reason, there was considerably more within-fluids variation concerning the buffering capacity against base than there was against acid.

The only significant difference in buffering capacity of the reproductive tract fluids, against acid as compared to base, occurred in the case of uterine fluid, which was more highly buffered against base than against acid.

The only statistically significant difference in the average buffering capacity against both acid and base was that cervicovaginal mucus had a lower buffering capacity than did the three other fluids.

For the sake of comparison, buffering capacity was determined on five samples of bovine blood serum. The blood was collected at the time of slaughter, allowed to clot, and centrifuged. Initial pH averaged about 7.9 and the average buffering capacity was 0.0126. The rather alkaline initial pH no doubt was due to a loss of CO_2 during handling. This loss of CO_2 probably also resulted in a lower buffering capacity than would normally be expected. As a matter of fact, the buffering capacity of follicular fluid against acid after the removal of CO_2 was determined to be 0.0067. Thus, it appears that carbonate accounts for at least 70% of the buffering capacity of follicular fluid.

The buffering capacity was also determined for five samples of seminal plasma obtained from three bulls. The initial pH was about 6.3 and the average buffering capacity against acid was 0.0260, and against base, it was 0.0183. Thus, it appears that female reproductive tract fluids and seminal plasma have buffering capacities which are comparable to or slightly greater than blood serum. Of course, hemoglobin contributes markedly to the buffering capacity of whole blood (1). Van Slyke (5) found the buffering capacity of whole human blood to be 0.0228.

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ALTERATION OF THE BOVINE ESTROUS CYCLE WITH OXYTOCIN[1]

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SUMMARY

The daily administration of natural or synthetic oxytocin to post-puberal nonlactating dairy heifers, during the first week of the estrous cycle, resulted in marked shortening of the diestrual period, the next heat occurring from eight to 12 days after the previous estrus, instead of the usual 21 days. These precocious heat periods appeared normal in every respect, being followed in all cases by ovulation at the expected time. In all cases, the subsequent diestrual periods were of normal lengths.

The corpora lutea formed during the oxytocin treatments failed to attain the expected size and contained a decreased number of normal luteal cells and an increased proportion of connective tissue elements. Concurrent injections of prolactin failed to overcome the diestrum-shortening effect of oxytocin. Daily progesterone injections prevented the oxytocin-treated heifers from returning to estrus until from four to six days after cessation of the progesterone injections. It was concluded that the observed effects of oxytocin were caused by inhibition of normal corpus luteum function, possibly by interfering with the secretion of a luteotrophic hormone from the anterior pituitary.

Oxytocin was less effective in causing diestrual shortening when either atropine or reserpine was administered concurrently, and was completely ineffective in inducing precocious estrus in hysterectomized, but not in sham-operated, heifers.

The importance of the hypothalamus in the regulation of anterior pituitary hormone secretion is well established (12), although the mechanisms by which this regulation is brought about are less well understood. The secretion of gonadotrophic hormones has been suggested by Benoit and Assenmacher (2) to be under the control of neurosecretory material, believed to be elaborated in hypothalamic nuclei and transported to the anterior pituitary by means of the hypophyseal portal blood vessels. The belief that this neurosecretory material contains the neurohypophyseal hormones, oxytocin and vasopressin (20), has led to the investigation of possible physiological roles which these hormones may play in the regulation of anterior pituitary hormone secretion.

Shibusawa *et al.* (21) have reported pitocin-induced alterations in urinary 17-ketosteroid excretion which they have interpreted as indicating that oxytocin induces gonadotrophic hormone secretion. Evidence for a close association between endogenous oxytocin release and gonadotrophin section has been reported in cattle. VanDemark and Hays (13, 24) have reported increased uterine motility and increased intramammary pressure, suggesting oxytocin output, in cows which received sexual stimulation in the form of manipulation of the

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reproductive tract per rectum, by the mere sight of the male, or by mounting and coitus. These observations can be correlated with those of Marion *et al.* (17), who showed that sexual stimulation in the form of sterile coitus caused cows to ovulate significantly earlier than when not mated, suggesting an earlier release of the ovulatory gonadotrophin as a result of mating. Preliminary work by Hansel, Armstrong, and McEntee (10) indicated that oxytocin injected at the onset of estrus caused cows to ovulate significantly earlier after the end of estrus than normally. The present experiments were conducted to further investigate the possible role of oxytocin in regulation of the secretion of gonadotrophic hormones by the bovine anterior pituitary.

EXPERIMENTAL PROCEDURES

Oxytocin injections were administered subcutaneously and/or intravenously once daily, beginning at various times during the estrous cycle, to normally cycling nonlactating dairy heifers, 1.5 to 3 yr. of age and weighing 270–365 kg. Injection regimens are listed in Table 1. Three different oxytocin-containing preparations were used, as follows: crude posterior pituitary extract (International Hormones, Inc., Brooklyn, N. Y.), containing 20 U.S.P. units oxytocin/ml and containing vasopressin as well; purified oxytocic principle (Armour, kindly supplied by Drs. I. Bunding and M. Davenport), containing 20 U.S.P. units per milliliter, and a synthetic polypeptide mixture (Syntocinon, Sandoz, kindly supplied by S. Gimpel and W. Newschwander), containing 10 U.S.P. units oxytocic activity/ml, but devoid of pressor activity.

Other treatments were superimposed upon oxytocin treatments in attempts to prevent the effects of oxytocin (as shown in Tables 1 and 2). Luteotrophin (Squibb) of ovine pituitary origin was administered subcutaneously at rates of 500 to 1,000 I.U. per day during oxytocin treatment. Progesterone was dissolved in corn oil and administered subcutaneously at the rate of 50 mg/day, beginning either on the day of estrus or the day after estrus and continued until 6, 10, or 14 days post-estrum. Atropine (Atropine sulfate, Merck) was administered subcutaneously on alternate days during oxytocin treatment, at dosages of 30 to 40 mg/kg. Reserpine (Serpasil, Ciba, kindly supplied by Dr. R. Gaunt) was administered either subcutaneously or intramuscularly at dosage levels of 2.5 to 5 mg/day during oxytocin treatment, with the exception of one heifer, which received only one injection of 10 mg. on the day of estrus, and one other heifer which received reserpine injections only on alternate days. Hysterectomies and sham hysterectomies were performed on the day after estrus, under light Equithesin [3] sedation and local anesthetization of the para-lumbar region, the site of the laparotomies. In hysterectomized heifers, the body and horns of the uteri were removed, the ovaries, oviducts, cervices, and vaginae being left intact. In sham-operated heifers, laparotomies were performed and the ovaries and uteri handled severely but left intact, with the exception of one uterine horn which was transected at the tubo-uterine junction in one heifer.

[3] Equi-thesin, brand of chloral hydrate, pentobarbital, magnesium sulfate mixture, Jensen-Salsbery Laboratories, Inc., Kansas City, Missouri.

TABLE 1

Effect of oxytocin administered alone or in combination with luteotrophin, atropine, reserpine, hysterectomy, or sham hysterectomy, on length of bovine estrous cycles

Experiment No.	No. of heifers	Daily oxytocin Dose [a]	Days post-estrum when injections begun	Duration of oxytocin treatment	Other treatment	Mean length of diestrum *(days)*		
						Control Period (a) prior to oxytocin	Period (b) during oxytocin treatment	Control Period (c) following oxytocin
		(U.S.P. units)		*(days)*				
1	5	150	15	15	None	22.4 [b]	10.6	20.4
2	12	50–150	0	7–10	None	20.2	11.3	20.8
3	6	100–150	1–7	7	None	21.5	15.7	20.5
4	2	100	0	7	Luteotrophin	19.0	8.5	20.0
5	6	100–150	0	7	Atropine	21.0	15.3	19.8
6	6	100–150	0	7	Reserpine	22.0	15.2	21.3
7	2	150	0	7	Hysterectomy	21.0	..... [c]	.....
8	2	150	0	7	Sham hysterectomy	22.5	12.5	14.0 [d]

[a] Oxytocin injected once daily at dosages of 50 U.S.P. units intravenously, 100 U.S.P. units subcutaneously, or 50 U.S.P. units intravenously plus 100 U.S.P. units subcutaneously.

[b] In Experiment 1, in which oxytocin treatments were begun on Day 15 of the cycle, Period (a) represents the diestrum during which treatments were begun.

[c] Hysterectomized heifers failed to return to estrus.

[d] Ovary of one heifer removed following precocious estrus, for histological examination. The resulting absence of corpus luteum caused this heifer to return to estrus again, eight days later.

TABLE 2

Effect of progesterone administered alone and concurrently with oxytocin on length of bovine estrous cycle

Heifer No.	Oxytocin treatment			Progesterone treatment			Length of diestrum	
	Dosage	Day of cycle when begun	Day of cycle when withdrawn	Dosage	Day of cycle when begun	Day of cycle when withdrawn	Prior to treatment	During treatment
	(U.S.P. units)			*(mg.)*				
131	None			50	1	10	21	21
135	None			50	1	14	21	19
127	150	0	6	50	0	6	20	10
142	150	0	6	50	1	10	20	14
141	150	0	6	50	1	10	22	14
137	150	0	6	50	1	14	20	18
138	150	0	6	50	1	14	26	20

All heifers were checked for estrus at least twice daily following the beginning of treatment. Heifers were considered to be in estrus if they would stand to be mounted by a bull or another heifer. Ovulations were determined by rectal palpations of the ovaries, and subsequent growth of the corpora lutea was followed in the same manner. Laparotomies were occasionally performed for visual examination of the ovaries, when thought necessary to confirm the observations of palpations. When one heifer was used for more than one treatment, at least one normal cycle was allowed to elapse between treatments.

RESULTS AND DISCUSSION

In Experiment 1 (Table 1), oxytocin injections were administered once daily, beginning at 15 days post-estrum, to determine if any alteration in the time of estrus could be induced as a result of this treatment. It was felt that a hastening of the onset of estrus would indicate a stimulation of secretion of the gonadotrophic hormones responsible for estrus and ovulation, presumably FSH and/or LH; whereas, any delay in the onset of estrus would indicate enhanced luteotrophin secretion. The observed normal diestrual lengths for Period a (Table 1) failed to indicate any enhancement of gonadotrophin or luteotrophin secretion by oxytocin. However, continuation of oxytocin treatments for an additional week after estrus resulted in the next estrus (Period b, Table 1) occurring from two to eight days after discontinuation of injections, or at an average of 10.6 days after the previous estrus. The duration of estrus and time of ovulation was within the normal range in all cases, and the next normal estrus occurred at the expected time, 20.4 days after the precocious estrus.

To determine which was the critical portion of the cycle, insofar as responding to oxytocin injections was concerned, injections were begun on the day of estrus in the next trial (Experiment 2, Table 1), and continued from seven to ten days. As in the first experiment, the majority of heifers exhibited precocious heat periods during or immediately following oxytocin treatment, with nine out of 12 heifers returning to heat in from seven to ten days after the previous estrus. The remaining three, however, did not return to heat until from 17 to 20 days. Two of the heifers which exhibited shortened cycles in Experiment 2 received injections of a synthetic oxytocic preparation (Syntocinon), making it appear likely that the observed effects were caused by oxytocin itself rather than by some impurity of neurohypophyseal origin. Two other heifers in this experiment returned to heat before discontinuation of injections, indicating that the precocious estrus did not depend upon the withdrawal of oxytocin. Of six heifers in which the beginning of oxytocin treatment was delayed until after ovulation (Experiment 3, Table 1), only two exhibited shortened diestrual periods (from eight to nine days), with the remainder ranging from 18 to 20 days.

It seemed improbable that the shortened diestrual periods were caused by a direct effect of oxytocin upon the ovaries without any alteration in the secretory pattern of anterior pituitary hormones, since the resulting estrous periods were of normal length and intensity and ovulations occurred at the normal time, approximately 10 hr. after the end of estrus. In addition, the next estrus oc-

curred approximately 21 days after the precocious estrus, rather than 21 days after the last previous normal estrus.

Rectal palpation of the ovaries of heifers during oxytocin treatment indicated that the corpora lutea of these heifers failed to attain a normal size. Corpora lutea of four of the oxytocin-treated heifers were removed at various times after the beginning of oxytocin treatments. Histological comparison of these corpora lutea with those removed from untreated heifers at comparable stages of the cycle revealed, in all instances, decreases in the size and number of normal luteal cells and marked increases in the proportion of connective tissue elements (see Figures 1 and 2). One of the four corpora lutea removed, and an additional one observed by means of laparotomy, contained a central cyst slightly less than 1 cm. in diameter.

Although it has been proposed that oxytocin treatment results in increased prolactin secretion in rats (3, 4, 6, 23), the observed shortening of the bovine estrous cycle by oxytocin in the present experiments suggested, instead, an inhi-

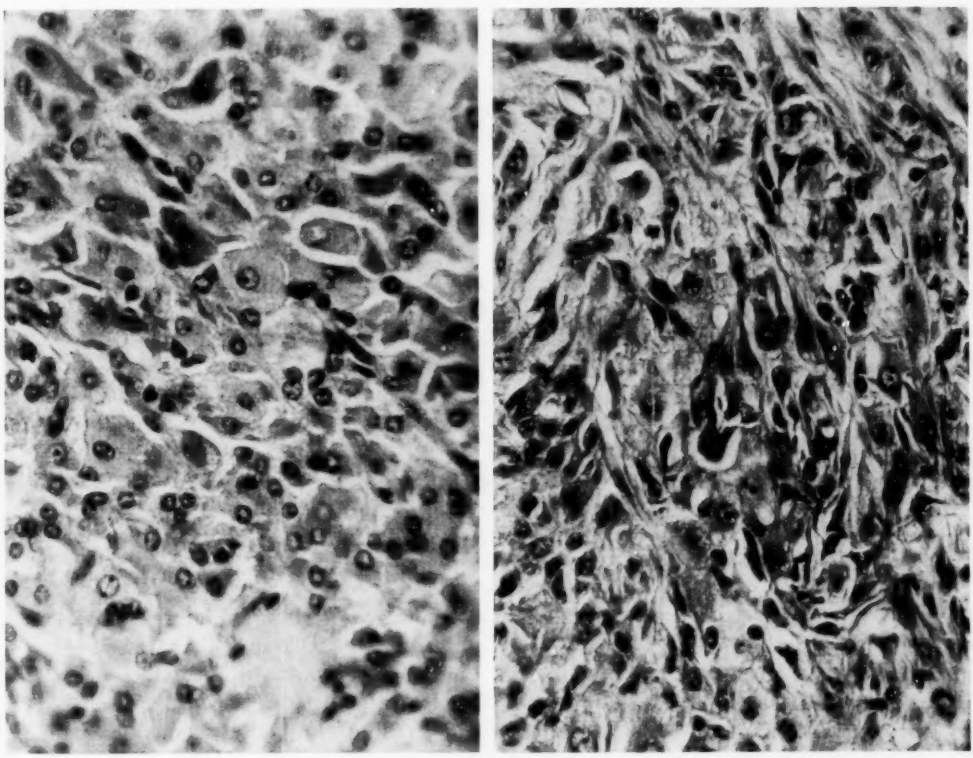

FIG. 1. Corpora lutea removed at seven days post-estrum from A, an untreated heifer and B, a heifer which had received seven daily oxytocin injections, beginning on the day of estrus. (Magnification 350 ×.)

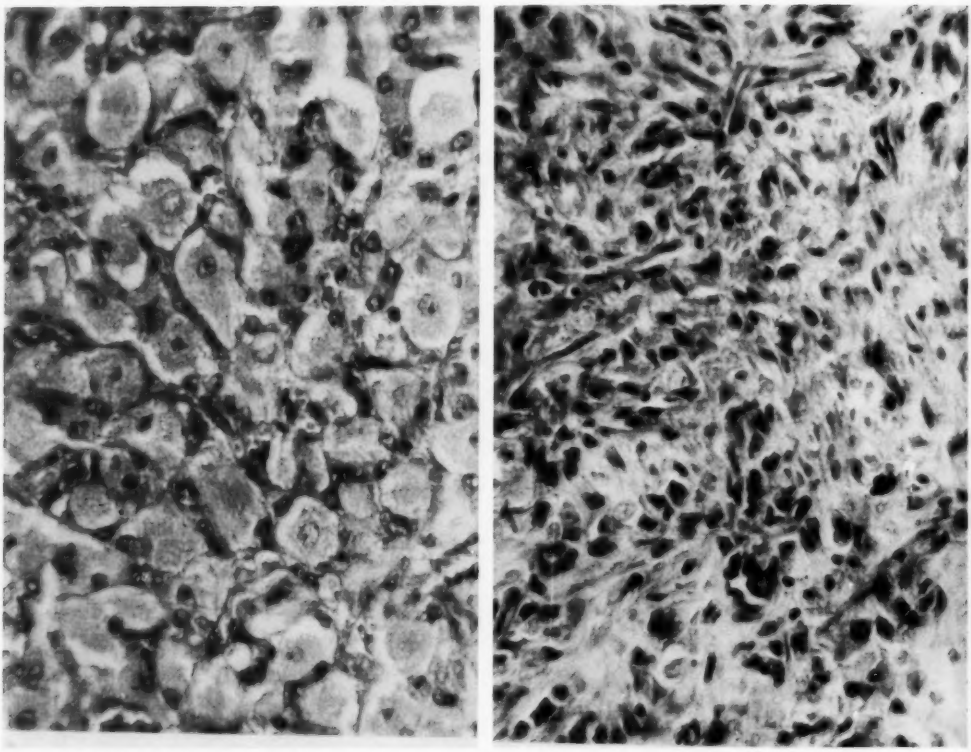

FIG. 2. A, Corpus luteum removed from an untreated heifer at 11 days post-estrum. B, Corpus luteum removed from an oxytocin-treated heifer ten days post-estrum. This heifer was in heat at the time of removal of the ovary. (Magnification 350 ×.)

bition of luteotrophic activity in this species. To determine if this resulted from a decreased secretion of prolactin, prolactin of ovine pituitary origin was administered concurrently with oxytocin, in an attempt to over-ride the diestrum-shortening effects of oxytocin. Prolactin failed to prevent the effect of oxytocin (Experiment 4, Table 1), although there is some doubt as to whether prolactin exerts luteotrophic action in the bovine, at least at the dosage levels used, as similar levels of prolactin failed to lengthen the estrous cycle in a heifer which received no other treatment. Smith, McShan, and Casida (22) also have reported the failure of prolactin injections to enhance luteal activity in the bovine.

From the observations that corpora lutea of oxytocin-treated heifers failed to attain normal size, as determined by rectal palpation, and that luteal cells failed to reach normal development, as revealed by histological examination, it seemed most likely that the shortening of the estrous cycle by oxytocin was the result of a failure of normal luteal activity, resulting in deficient progesterone secretion. To test this hypothesis, progesterone was administered to five heifers

for varying periods of time during and following a seven-day oxytocin regimen. As shown in Table 2, when oxytocin was administered during the first seven days of the cycle along with progesterone, all heifers returned to heat from four to six days following withdrawal of progesterone, whether progesterone treatments were discontinued at six, ten, or 14 days post-estrum. Two heifers which received progesterone alone during the first half of the estrus cycle exhibited cycles of normal length. From these experiments, it appears likely that the early return to heat following oxytocin treatment is not caused by an ability of oxytocin to induce gonadotrophin secretion directly, but rather is caused by an inhibition of normal corpus luteum function, with the resulting progesterone deficiency permitting the anterior pituitary to secrete the estrus-inducing gonadotrophins earlier in the cycle than normal.

It remains to be determined whether the observed diestrual shortening was the result of direct effects of oxytocin upon the cells of the anterior pituitary, or of indirect effects on the pituitary mediated via some nervous reflex, presumably involving the hypothalamus. In an attempt to determine if the nervous system was involved in the observed responses, oxytocin treatments were combined with atropine and reserpine treatments in Experiments 4 and 5, respectively. The anticholinergic drug, atropine, has been found to block ovulation in the cow as well as in other species (11), and the tranquilizing drug, reserpine, has been shown to prevent ovulation in rats (1). The effects of both of these drugs are believed to be mediated through the central nervous system. In the present experiments, three out of six heifers which received both atropine and oxytocin returned to heat in from ten to 13 days (Experiment 3, Table 1); whereas, the other three exhibited cycles of normal length (19–21 days). Two of the six heifers receiving reserpine (Experiment 6, Table 1) plus oxytocin exhibited diestrual periods of nine and ten days, whereas one other heifer returned to heat in 15 days after the previous estrus. The remaining three heifers had diestrual periods of 19 days.

The apparent decreased ability of oxytocin to cause diestrual shortening in heifers treated with these drugs tends to implicate the nervous system in the observed responses to oxytocin, although direct inhibitory effects of atropine and reserpine upon the anterior pituitary can not be ruled out. Hence, it can not be stated with certainty that the effect of oxytocin in inducing precocious estrus is mediated via the nervous system.

Because of the possibility that atropine and reserpine might have prevented afferent nerve impulses from reaching the hypothalamus, it became of interest to determine if oxytocin-induced contractions of the uterus might be the source of these afferent impulses. To test this hypothesis, two heifers were hysterectomized the day following estrus, and oxytocin was administered for seven days, beginning on the day of estrus, as in previous experiments. Two additional heifers were subjected to sham operations, but were otherwise treated the same. Neither of the hysterectomized heifers exhibited shortened estrous cycles in response to oxytocin (see Table 1, Experiment 7). The ability of oxytocin to shorten the diestrual periods in sham-operated heifers (Table 1, Experiment 8)

makes it evident that the anesthesia and surgery themselves did not prevent the action of oxytocin.

Although both sham-operated heifers continued to exhibit normal estrous cycles following the operations, neither of the hysterectomized heifers resumed cyclic sexual activity, thus confirming the previously reported observations of Wiltbank and Casida (25). One of the hysterectomized heifers was administered oxytocin injections for an additional seven days, beginning 61 days after the last heat period, to determine if this treatment would induce estrus and ovulation after a suitable time had elapsed to allow recovery from the operation. Again, no estrus or ovulation resulted. Forty days after hysterectomy, the other heifer was injected with 50 mg. FSH (Pituitary Follicle Stimulating Hormone, Armour), followed by an injection of 10,000 I.U. HCG (Human Chorionic Gonadotrophin, Squibb) four days later, to determine if the ovaries were capable of responding to gonadotrophic stimulation in the absence of the uterus. Although no psychic manifestations of estrus occurred, several palpable follicles developed in response to the FSH treatment, most of which ovulated and developed into large corpora lutea following HCG treatment. These corpora lutea persisted and again the heifer remained anestrous for at least 36 days, at which time one ovary was removed for histological examination. This ovary contained several well-developed corpora lutea, as well as three large unovulated follicles which were heavily luteinized.

While it is possible that oxytocin itself exerts a direct inhibitory action upon the corpus luteum, it would appear more likely that the effects are mediated indirectly via the anterior pituitary. If the oxytocin preparations stimulated the release of ACTH in the present experiments, the resulting excessive secretion of adrenal corticosteroids possibly might have resulted in inhibition of luteal function. However, this sems unlikely, as four additional heifers which were injected with 20 to 40 mg/day of epinephrine for seven days, and one heifer which received 100 mg/day of ACTH (Armour) for seven days, exhibited no alteration in estrous cycle length.

A more likely explanation is that oxytocin causes an inhibition of secretion by the anterior pituitary of a luteotrophic hormone, the identity of which can not be stated at the present time. At least two possible mechanisms by which such an inhibition may be brought about seem worthy of consideration and further investigation. If endogenous oxytocin is the neurohumoral stimulator of luteotrophic hormone secretion, as has been proposed by Benson and Folley (3) for the rat, one might expect large doses of exogenous oxytocin to exert similar effects. However, because of the possibility of endogenous oxytocin reaching the anterior pituitary by means of direct vascular connections between the posterior and anterior lobes, as have been described for the rat (5, 16), and the dog (14), or directly from the hypothalamus by the hypophyseal portal vessels, it is conceivable that endogenous oxytocin may reach the anterior pituitary in a much more concentrated state than ever could be achieved by injections of exogenous oxytocin. Thus, exogenous oxytocin treatments may be below physiological levels as far as the anterior pituitary is concerned, and yet be above

physiological levels as far as uterine contractions and milk ejection are concerned. This excess of exogenous oxytocin may then act to inhibit the release of endogenous oxytocin, the lack of which results in failure of stimulation of luteotrophic hormone release.

Evidence that repeated treatment of cows with exogenous oxytocin does indeed depress the release of endogenous oxytocin has been presented by Knodt and Petersen (15). These workers showed that continued treatment of cows with exogenous oxytocin resulted in a progressive decrease in the amount of normal milk that could be obtained at a milking, and an increase in the amount of residual, or pitocin, milk which could only be obtained following oxytocin injection.

An alternative explanation for the mechanism by which exogenous oxytocin inhibits normal corpus luteum development may be that the anterior pituitary secretes luteotrophic hormone in the absence of any neural or neurohumoral activation, and that in order for luteotrophin secretion to stop, some neural deactivation mechanism must be brought into play. Evidence that this may indeed be true, at least in the rat, has been provided by the experiments of Everett and coworkers (8, 9, 18, 19) and Desclin (7), showing that anterior pituitary transplants in sites other than under the median eminence enabled, or even favored, the persistence of corpora lutea for long periods of time. Retransplantation of the pituitaries under the median eminence resulted in corpus luteum regression and resumption of cyclic activity, provided that the pituitaries became revascularized by the hypophyseal portal vessels. Oxytocin itself, or some other neurohumoral substance released in response to central or peripheral actions of oxytocin, may then be carried by the hypophyseal portal vessels to the anterior pituitary, where it inhibits further luteotrophic hormone secretion, thereby causing corpus luteum involution.

The present evidence does not define which of these possibilities, if either, is the means by which oxytocin shortens the bovine estrous cycle, and further research is under way to attempt to establish the physiological role which oxytocin may play in estrous cycle regulation.

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OBSERVATIONS ON THE ESTROUS CYCLES OF REPEAT-BREEDING DAIRY HEIFERS[1]

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SUMMARY

The average duration of heat for 97 estrous periods of 17 repeat-breeding heifers was 19 ± 4 hr. Ovulation occurred 12 ± 3 hr. after the end of heat. Failure in ovulation occurred in 18% of the estrous cycles. The repeatability for duration of heat was about 16%, but the repeatability for time of ovulation was almost zero. There was no significant correlation between duration of heat and time of ovulation.

It seems reasonable that repeat breeding in cows could be due to irregularities of the estrous cycle, which would make it more difficult to choose the proper time of service. However, several studies have shown that time of breeding, within rather broad limits, is not very important in determining whether conception occurs in first-service cows bred artificially (1, 2, 3). Roberts (4) summarized the literature regarding failure of fertilization and concluded that fertilization does not occur in about 40% of the repeat-breeder cows; whereas, in virgin heifers bred at first service only about 15% are not fertilized. Although there are undoubtedly many possible causes for fertilization failure, it seems likely that one of the causes could be either improper timing of service due to irregularities in the estrous cycle and time of ovulation, or to inability of spermatozoa to live a normal length of time in the female reproductive organs.

The present study was made to determine whether repeat-breeding heifers were likely to have abnormalities in the duration of heat and time of ovulation.

METHODS

Seventeen repeat-breeding heifers were obtained from dairymen in central Kentucky. According to the owners, these animals averaged about 2.5 yr. of age (14 mo. to 4 yr.) and had been bred an average of 5.8 times (range four to 13) without becoming pregnant. Upon clinical examination, none of the heifers had any recognizable abnormalities, except one case of double cervix, one of partial hymen, and one of granular vaginitis. In two animals, slight ovarian adhesions were found at slaughter.

The heifers were checked every 8 hr. for signs of heat. Though other signs were used as aids, an animal was considered to be in heat only when it would stand to be mounted by other heifers. After heat started, the animals were checked every 4 hr. for the end of heat. After the end of heat, they were palpated rectally every 2 hr., to determine the time of ovulation. Data were obtained for a total of 97 estrous periods, or an average of 5.7 periods per heifer.

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RESULTS AND DISCUSSION

The average duration of heat for 97 periods of 17 repeat-breeding dairy heifers was 19 ± 4 hr. and the average time of ovulation was 12 ± 3 hr. after the end of heat. These values compare very favorably with the values for normal animals as cited by Roberts (4). Analysis of variance indicated that individual heifers had a tendency to have about the same duration of heat at successive estrous periods. As shown in Table 1, there was significantly more variation among heifers than there was among periods of the same heifers ($P < 0.01$). The repeatability for duration of heat was about 0.16.

Regarding time of ovulation, analysis of variance (Table 2) indicated that there was about as much variation among ovulation times for the same heifers as there was among ovulation times for different heifers. The repeatability for time of ovulation was $-.0018$ which is, for all practical purposes, zero.

The correlation coefficient between duration of heat and time of ovulation was .074 which, of course, was not significant.

TABLE 1

Analysis of variance regarding duration of heat

Source	Degrees of freedom	Sum of squares	Mean squares
Total	96	1,713.0	
Among heifers	16	505.9	31.62
Within heifers	80	1,207.1	15.09*

* $P < 0.01$.

TABLE 2

Analysis of variance regarding time of ovulation

Source	Degrees of freedom	Sum of squares	Mean square
Total	76	839.6	
Among heifers	15	164.5	10.97
Within heifers	61	675.1	11.07

There were 17 cases in which ovluation apparently did not occur. This represented 18.0% of the estrous periods. There was no indication that ovulation failure had any marked effect on the time at which the next estrous period occurred.

These data seem to indicate that abnormalities in the duration of heat and time of ovulation did not contribute to the infertility of these repeat-breeding heifers. However, ovulation failure may have been somewhat more important than has been indicated by previous studies, in which only 3–5% of the repeat breeders failed to ovulate (4).

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NORMAL STANDARDS, REPEATABILITY, AND PRECISION OF PHENOLSULFONPHTHALEIN CLEARANCE MEASURES FOR RENAL FUNCTION IN DAIRY CATTLE[1]

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SUMMARY

Twenty-eight dairy bull calves and 24 dairy cows were used in trials to determine normal standards for phenolsulfonphthalein (PSP) fractional clearance, PSP volume of distribution, and PSP volume clearance as measures of renal function. The mean PSP fractional clearances per minute were 0.0250 and 0.0431, the PSP volumes of distribution were 31.3 and 19.0% of body weight, and the corresponding PSP volume clearances as milliliters per kilogram of body weight were 7.48 and 8.00 in calves and cows, respectively.

The repeatability and precision of the various PSP clearance measures were low, as determined from variability found in trials conducted in 20 bull calves and 22 cows on two successive days. The reason for this was not determined.

It was suggested that both PSP fractional clearance and PSP volume clearance measures should be used as criteria of renal function.

Mixner and Anderson (5) have presented phenolsulfonphthalein (PSP) fractional clearance as a measure of renal function in dairy animals. This procedure involves the measurement of the rate of disappearance of PSP from blood plasma after its intravenous infusion and does not require the collection of urine samples. The level of PSP in the plasma was shown to decrease exponentially with time following its injection, in which the $\log_e$ of PSP concentration in the plasma was inversely and rectilinearly related to time.

Goodman and Kingsley (4), in a study of hepatic function in humans, combined the measure of plasma sulfobromphthalein fractional clearance and the measure of the volume of distribution of sulfobromphthalein to calculate sulfobromphthalein volume clearance (milliliter of plasma cleared per minute). This last measure of hepatic function is equivalent to the usual plasma clearance measure as used for renal function (6).

The purpose of this paper is to present normal standards for, and to establish the repeatability and precision of the PSP clearance measures in, dairy cattle. A preliminary report has been made on this study (1).

EXPERIMENTAL PROCEDURES

The basic procedures used for determining PSP clearance measures were those outlined by Mixner and Anderson (5). The two main criteria of renal function were the PSP fractional clearance per minute and the PSP volume clearance as milliliter of plasma cleared per minute per kilogram of body weight

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The PSP volume clearance as milliliter per minute was calculated by multiplying the PSP fractional clearance per minute by the PSP volume of distribution in milliliters. The PSP volume of distribution (as per cent of body weight) will be presented also, since it is a component of the PSP volume clearance measure.

Normal standards for renal function were developed from data obtained on 28 Holstein and Guernsey bull calves which were six to 14 days of age, and on 24 Holstein and Guernsey cows which were in advanced lactation or dry. Data were used from a single trial-run on each animal. The mean was used as the measure of the central tendency, and the standard deviation, the coefficient of variation, and the range of values were used as measures of dispersion or distribution.

Repeatability estimates of the various PSP clearance measures were obtained by running the clearance test on two successive days in 20 bull calves and 22 cows which were part of the group used to determine normal standards. An analysis of variance was made on the data to determine the significance of animal effects. Estimated variances also were calculated and the estimated per cent of total variance due to animal effects was used as an index of repeatability of the various measures. The mean squares or estimated variances for determinations within animal were used to calculate the standard error of a single determination and the coefficient of variation of a single determination as measures of precision.

In addition, zero-order coefficients of correlation were calculated on an among-animal basis on the repeatability data, to indicate the basic interrelations existing among the primary measures of renal function.

RESULTS AND DISCUSSION

Normal standards of PSP clearance measures for calves and cows are presented (Table 1). There were statistical differences between calves and cows

TABLE 1

Mean normal standards for PSP clearance measures

Characteristic	Bull calves	Cows
No. of animals	28	24
Body weight *(kg.)*	42.0	589
PSP fractional clearance per minute	0.0250	0.0431
Standard error of mean	0.0010	0.0012
Standard deviation	0.0053	0.0060
Standard error of standard deviation	0.0007	0.0009
Coefficient of variation *(%)*	21.0	14.0
Range	0.0142 to 0.0342	0.0322 to 0.0548
PSP volume of distribution *(% of body weight)*	31.3	19.0
Standard error of mean	2.2	1.2
Standard deviation	11.5	5.7
Standard error of standard deviation	1.5	0.8
Coefficient of variation *(%)*	36.7	30.0
Range	6.8 to 56.5	10.7 to 29.6
PSP volume cleraance *(milliliters per minute per kilogram of body weight)*	7.48	8.00
Standard error of mean	0.56	0.36
Standard deviation	3.00	1.76
Standard error of standard deviation	0.40	0.25
Coefficient of variation *(%)*	39.5	22.0
Range	2.28 to 13.2	4.70 to 10.9

with respect to PSP fractional clearance per minute and PSP volume of distribution as per cent of body weight ($P < 0.01$), but not for PSP volume clearance as milliliters per kilogram of body weight ($P > 0.05$). Calves had lower PSP fractional clearance and higher PSP volume of distribution as per cent of body weight than did cows, which compensated for each other to give similar values in calves and cows for PSP volume clearance as milliliters per minute per kilogram of body weight. Actually, it would seem that PSP volume clearance in milliliters per minute in calves and cows should vary directly with some power of body weight, such as the 0.73th power, which is the way that basal metabolic rate varies with body weight in mature animals of different species (3).

The analysis of variance of among-animal differences in PSP clearance measures (Table 2) indicated that in both calves and cows there were significant animal differences in PSP volume clearance in milliliters per minute per kilogram of body weight. Only in the calves were there significant animal differences in PSP fractional clearance or in PSP volume of distribution as per cent of body weight.

The repeatability of PSP clearance measures based on per cent of total variance varied from 17.7 to 65.7% for PSP volume of distribution as per cent

TABLE 2

Analysis of variance and estimated variances for PSP clearance measures in 20 bull calves and 22 cows[a]

Type of animal, renal clearance measures, and sources of variation	Degrees of freedom	Mean square	Estimated variances	
			σ-2	%
Bull calves				
PSP fractional clearance				
Among calves	19	0.0000395[b]	0.0000142	55.9
Determinations in calves	20	0.0000112	0.0000112	44.1
PSP volume of distribution *(% of body weight)*				
Among calves	19	277.6[b]	100.1	65.7
Determinations in calves	20	57.4	57.4	34.3
PSP volume clearance *(milliliters per minute per kilogram body weight)*				
Among calves	19	13.50[b]	4.95	57.8
Determinations in calves	20	3.61	3.61	42.2
Cows				
PSP fractional clearance				
Among cows	21	0.0000587	0.0000142	31.9
Determinations in cows	22	0.0000303	0.0000303	68.1
PSP volume of distribution *(% of body weight)*				
Among cows	21	29.2	4.4	17.7
Determinations in cows	22	20.4	20.4	82.3
PSP volume clearance *(milliliters per minute per kilogram of body weight)*				
Among cows	21	4.73[b]	1.58	50.1
Determinations in cows	22	1.57	1.57	49.9

[a] Determinations in animals based on trials conducted on two successive days.
[b] Statistically significant ($P < 0.01$).

of body weight in cows and calves, respectively (Table 2). The causes of these low repeatabilities are not known. They may represent random variation or they may be concerned with the precision of the assay procedures. This problem is being further investigated.

Estimates of the precision with which PSP clearance measures may be made are presented (Table 3). These are the standard error of a single determination and the coefficient of variation of a single determination based on the error term variance (determinations in calves and cows) of Table 2. In general, the coefficients of variation (range of 12.7 to 24.7%) are considered high and the precision of individual determinations somewhat poor.

It was interesting to determine how the various components of the PSP clearance were interrelated. Zero-order coefficients of correlation on an among-animal basis were calculated between the various measures, using the repeatability data for 20 bull calves and 20 cows (Table 4). These correlations pose the question as to whether PSP fractional clearance or PSP volume clearance would be the better measure of renal function, since in normal animals, at least, they have little correlation to each other. Anderson and Mixner (2) reported that the injection of Synthalin A in dairy bull calves caused a marked and significant decrease in PSP fractional clearance. Not reported was the fact

TABLE 3

Precision of PSP clearance measures as determined from error term variance in Table 2

Item	Calves	Cows
Number of animals		
Mean PSP fractional clearance	0.0256	0.0435
S.E. of a single determination	0.0034	0.0055
C. V. of a single determination *(%)*	13.1	12.7
Mean PSP volume of distribution *(% of body weight)*	31.8	18.2
S.E. of a single determination	7.6	4.5
C.V. of a single determination *(%)*	23.9	24.7
Mean PSP volume clearance *(milliliters per minute per kilogram of body weight)*	7.89	7.77
S.E. of a single determination	1.90	1.25
C.V. of a single determination *(%)*	24.1	16.2

TABLE 4

Coefficients of correlation among PSP clearance measures in 20 bull calves and 22 cows on among-animal basis

Characteristics correlated	Coefficients of correlation	
	Calves	Cows
PSP fractional clearance *versus* PSP volume of distribution in milliliters	−.52*	−.56**
PSP fractional clearance *versus* PSP volume clearance in milliliters per minute	−.15	−.06
PSP volume of distribution in milliliters *versus* PSP volume clearance in milliliters per minute	.90**	.85**

* and ** indicate statistical significance, $P < 0.05$ and $P < 0.01$, respectively.

that there was no change in PSP volume of distribution, but a marked decrease in PSP volume clearance occurred. It is conceivable that some disease condition or toxic substance might also cause a change in PSP volume of distribution. Thus, it is the feeling of the authors at this time that although PSP fractional clearance might suffice in most instances as a measure of renal function, a more complete picture of the situation would be obtained from PSP fractional clearance, PSP volume of distribution, and PSP volume clearance measures. From the viewpoint of renal physiology, the PSP volume clearance is the more complete measure of renal function, for the values may be compared in an absolute way with conventional clearance values obtained by usual methods of plasma and urine analysis (6).

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TECHNICAL NOTES

ELECTROPHORESIS OF UNFRACTIONATED PROTEINS FROM HOMOGENIZED WHOLE MILK

Conventional electrophoresis of milk proteins by the moving boundary technique of Tiselius (3) requires that the suspension be relatively free of lipid material. It was shown by Tobias *et al.* (4) that when the ether extract of the undiluted suspension was greater than 0.08%, the photographs of electrophoretic patterns were partially overexposed and partially underexposed. With ether extract contents greater than 0.12% it becomes very difficult, if not impossible, to obtain well-defined photographs.

It was observed that fat is difficult to remove from homogenized milk without denaturing the proteins. Ordinary centrifugation can not be depended upon, as the force necessary to separate the fat must be of such magnitude that some of the casein is sedimented at the same time. The use of organic solvents is undesirable because of the possible deleterious effect on proteins. Demulsifying agents (2), for the most part, require heating to elevated temperatures to produce effective fat sparation, and may cause additional unwanted changes in the proteins. Filtration is generally unsuccessful, as the necessarily small filter pores are readily clogged by the fat globules, thus preventing the flow of fat-free serum through the filter.

A technique is described here by which it is possible to obtain a fat-free skimmilk from homogenized whole milk by application of centrifugal force.

Experimental Procedure

A 34-ml. sample of homogenized milk was diluted to 85 ml. with 0.1 *M* NaCl, giving a dilution ratio of 2.5 to 1. The diluted sample dialyzed against 2,000 ml. of 0.1 *M* NaCl for 30 hr. at a temperature of 0–4° C. A mechanical dialyzer as described by Reiner and Fenichel (1) was employed, using Visking seamless cellulose tubing as the membrane. Centrifugation was performed in a Spinco Model L preparatory ultracentrifuge, using Rotor No. 21 and 100-ml. capacity centrifuge tubes.

The dialyzed milk was tempered to 37° C., transferred to the centrifuge tube, and centrifuged at 20,000 r.p.m. (40,000 × G) for 30 min. at 37° C. It required approximately 10 min. to attain desired speed, and 20–30 min. to come to a stop after the power was turned off. The fat was found deposited in a thick, creamy layer in the upper corner of the tube. It was readily redispersible, which necessitated cautious handling during the removal of fat-free serum. Best results were obtained with a thin, pointless hypodermic needle inserted into the tube slowly and without jerky movements while the tubes were still in the rotor. Twenty milliliters of the fat-free serum was dialyzed against 750 ml. of veronal buffer of pH 8.7 and 0.1 μ in a mechanical dialyzer prior to electrophoresis.

Electrophoresis was carried out in a Perkin-Elmer Tiselius electrophoresis apparatus equipped with a Philpot-Svensson cylindrical lens.

Data are presented for homogenized skimmilk and homogenized whole milk, both subjected to the ultracentrifuge treatment, and homogenized skimmilk which was not ultracentrifuged. The raw milk as received from the dairy farm was warmed to 90° F. and a portion of it was separated three times in a conventional cream separator. The skimmilk and the whole milk then were pasteurized by heating to 161° F. for 15 sec. in jacketed steam kettles, cooled to 125° F., homogenized at 2,500 p.s.i., and cooled to 40° F. over a surface cooler.

Results

Results of electrophoretic analyses are given in Table 1 and the patterns are shown in Figure 1. Mobilities in all cases were measured from the false boundary and the relative areas were obtained by means of a planimeter. Corrections were made for the δ and ϵ effects.

TABLE 1

Effect of centrifugation of dialyzed whole milk and skimmilk on the electrophoretic properties of its proteins

Component	Ascending		Descending	
	Mobility	Relative area	Mobility	Relative area
	($cm^2\ sec^{-1}\ v^{-1} \times 10^5$)	(%)	($cm^2\ sec^{-1}\ v^{-1} \times 10^5$)	(%)
	Homogenized skimmilk			
γ-casein	−3.16	1.1	−2.01	2.3
β-casein	−4.32	25.2	−3.27	19.3
β-lactoglobulin	−5.63	8.9	−4.38	7.4
α-casein	−6.71	64.8	−5.84	70.9
	Ultracentrifuged homogenized skimmilk			
γ-casein	−3.15	2.5	−2.00	1.3
β-casein	−4.36	25.6	−3.33	20.9
β-lactoglobulin	−5.75	8.9	−4.48	7.9
α-casein	−6.78	63.0	−5.94	69.7
	Ultracentrifuged homogenized whole milk			
γ-casein	−3.15	2.6	−2.00	1.4
β-casein	−4.36	27.2	−3.33	18.6
β-lactoglobulin	−5.63	6.2	−4.48	8.8
α-casein	−6.78	63.9	−5.93	71.2

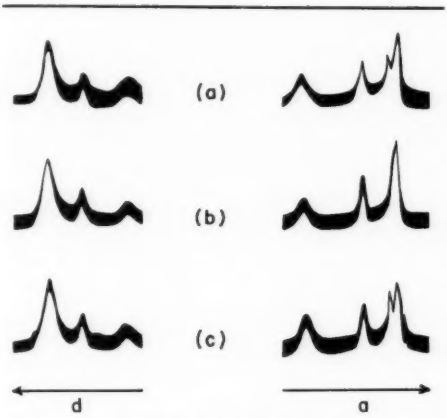

FIG. 1. Effect of centrifugation of dialyzed whole milk and skimmilk on the electrophoretic patterns. (a) Homogenized skimmilk, (b) ultracentrifuged homogenized skimmilk, (c) ultracentrifuged homogenized whole milk. Veronal buffer of pH 8.4 and 0.1 μ. Electrophoresis was carried out for 3,600 sec. with a current of 12.5 mA.

It can be seen that the mobilities of each of the components are within limits of experimental error not affected by the treatment of the samples. The same is true for the relative areas. The pattern of the uncentrifuged skimmilk proteins, Figure 1 (a), shows evidence of over- and under-exposure, which indicates that this sample had a higher lipid content than the two centrifuged samples. Over-exposure is indicated by thickening of the curves. The α-casein peaks in the ascending patterns exhibited considerable inhomogeneity, a phenomenon observed previously (5). The different appearance of the α-casein peak in the ascending pattern of centrifuged skimmilk is believed not to be due to the experimental treatment of the sample. The effect was also noted in other samples, and no experimental treatment was found to give it reproducibly.

DISCUSSION

To establish the optimum temperature at which centrifugation should be conducted, replicate samples were centrifuged at 5 and 37° C. No differences were observed in the electrophoretic properties of the proteins under these two conditions, but there were some differences in the appearance of the fat-free serum. The serum obtained at 5° C. was generally somewhat cloudy and exhibited some tendency to foam. The electrophoretic pattern on the ascending side was under-exposed under conditions which produced well-defined patterns on the descending side. Only the portion of the descending pattern past the α-casein peak was under-exposed. Ultracentrifugation at 37° C. was chosen because no turbidity developed and both patterns were well-defined.

The electrophoretic patterns of the proteins of homogenized milks showed a small component moving just ahead of α-casein. In most cases, as illustrated in Figure 2, this component could be observed both in the ascending and in the descending patterns and, thus, did not appear to be the same constituent which is generally observed as an asymmetry in the ascending pattern of α-casein. Further studies will be necessary to determine whether this is a true protein component or an anomaly. In this study, the new component was considered part of the α-casein complex, in determining its relative area.

FIG. 2. Electrophoretic patterns of ultracentrifuged, dialyzed whole milk, showing fast-moving component. Veronal buffer of pH 8.4 and 0.1 μ. Electrophoresis was carried out for 3,600 sec. with a current of 13 mA.

Various centrifugation speeds were tried, but consistently good fat removal could be obtained only at 20,000 r.p.m. for 30 min. Dialysis against NaCl solution prior to centrifuging insured that the casein remained in suspension. Otherwise, under these conditions, sedimentation of casein would have been observed. It is possible that the angle head of an International centrifuge might be adequate in removing the fat, but no attempts to use it have been made in this study.

An examination of the patterns in Figure 1 would indicate that the whey proteins did not resolve. However, when enlarged tracings of the patterns were made on graph paper it became apparent that there was an area between α-casein and β-casein which could not be accounted for by these two components. The component responsible for this area had a mobility and relative concentration which normally would be obtained from β-lactoglobulin in unfractionated milk. Hence, it was reported as such in Table 1.

Upon dialysis of the 85 ml. of the diluted sample against 0.1 *M* NaCl, the volume increased to 102 ml. which, in effect, gave a 3-to-1 dilution of the original milk prior to centrifugation. No further increase in volume was observed upon the second dialysis against veronal buffer prior to electrophoresis.

SUMMARY

A procedure is described consisting of dialysis of homogenized whole milk against 0.1 *M* NaCl and ultracentrifugation at 40,000 × G, by which it is possible to obtain a suspension

of unfractionated milk proteins suitable for electrophoretic analysis. No changes in mobilities or relative concentrations of electrophoretic components at pH 8.7 and 0.1 μ could be attributed to this manner of sample preparation.

A question is raised as to the identity of a small peak observed in the electrophoretic patterns of homogenized whole milk which moved with a slightly higher mobility than α-casein.

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DETERMINATION OF PHOSPHORUS IN MILK LIPIDS

Investigation of milk lipids often requires determination of the phosphorus in residues from extracts such as those obtained by the Mojonnier procedure or from chromatographic eluates. Colorimetric procedures generally are preferred to titrimetric or gravimetric methods, as being faster and better adapted to analyzing small amounts of material. In our laboratory we have used a modification of the method of Harris and Popat (2) for determining phosphorus in cottonseed lipids. The method employs perchloric acid for sample digestion and *p*-methylaminophenol sulfate (elon) as a reducing agent for color development. Both of these steps are performed in the same test tube, to avoid transfer losses.

In developing their method, Harris and Popat investigated the effect of a number of variables on the digestion of lipid samples and on color development with elon. They stated that the oxidation and digestion procedure can be completed within 5 min. However, they did not report any data showing the effect of varying the time of digestion, although they emphasized that the final clarification step in the digestion procedure must be complete. Furthermore, no data were presented on the recovery of organic phosphorus. When various individuals in our laboratory used Harris and Popat's original procedure to determine phosphorus in milk lipids and in a variety of phospholipid samples, the results often were erratic. Subsequent investigation has shown that results are more consistent and accurate when a longer digestion period is employed. The modified procedure is described below.

Samples are prepared by pipetting volumes of lipid solutions of known concentrations into 35-ml. Pyrex test tubes calibrated at 20.0 ml. and containing two glass beads. Solvents are evaporated with a water bath at 70° C. and 1.0 ml. of 72% perchloric acid is added to each tube. To avoid explosion hazard, the lipid weight should not exceed 75 mg. for this amount of perchloric acid. Oxidation is initiated by adding two drops of nitric acid immediately before gently heating each tube (held in a test-tube holder) by hand over a micro-burner. The reaction mixture should be agitated continuously until foaming subsides and it boils evenly. Under these conditions, the rate of oxidation can be controlled readily. Then the mixture is heated more strongly until it becomes clear and essen-

TABLE 1

Effect of digestion time on determination of lipid phosphorus

Sample	Weight	Digestion time	Absorbance	Phosphorus
	(mg.)	*(min.)*		*(%)*
Milk Lipid A	10.4	0	0.235	0.357
	10.4	6	0.258	0.392
	10.4	9	0.267	0.406
	10.4	12	0.267	0.406
	10.4	15	0.275	0.418
	10.4	18	0.271	0.412
	10.4	20	0.267	0.406
	4.16	20	0.107	0.406
	6.24	20	0.162	0.410
	8.32	20	0.221	0.410
Milk Lipid B	2.64	0	0.370	2.22
	2.64	6	0.505	3.03
	2.64	9	0.550	3.30
	2.64	12	0.555	3.33
	2.64	15	0.559	3.35
	2.64	18	0.553	3.32
	2.64	21	0.558	3.35
Dimyristoyl lecithin [a]	2.10	0	0.407	3.08
	1.05	1	0.224	3.37
	1.05	2	0.266	4.00
	1.05	3	0.291	4.38
	0.86	9	0.242	4.43
	1.73	9	0.482	4.40
	0.86	12	0.244	4.46
	1.73	15	0.494	4.52
	1.73	21	0.488	4.46

[a] LaMotte Chemical Products Co., Chestertown, Maryland.

tially colorless, and white fumes of perchloric acid appear.

After the above oxidation, two more drops of nitric acid are added to each tube. Then they are placed in a micro-Kjeldahl digestion rack and the samples digested for 20 min. with sufficient heat to boil them gently without appreciable loss of perchloric acid. After cooling and diluting with about 15 ml. of water, each sample is mixed with 1.0 ml. of 5% ammonium molybdate solution. Then 2.0 ml. of elon solution (0.5 g. *p*-methylaminophenol sulfate, 12.5 g. sodium bisulfite, and 2.4 g. sodium sulfite made up to 100 ml.) is added and the mixture diluted to 20.0 ml. The absorbance (optical density) at 820 mμ of each sample is read against a blank 15 min. after the elon addition, and the percentage of phosphorus is obtained from a standard curve in the usual way.

Table 1 illustrates the effect of time of digestion on lipid phosphorus recovery. The samples required at least 12 min. of digestion after the initial major oxidation steps. On the basis of these results, and subsequent experience with samples ranging up to 60 mg., the standard digestion period of 20 min. was adopted. The results for the pure synthetic L-α-(dimyristoyl)-lecithin check with the theoretical value of 4.46%. This sample contained 4.40 and 4.41% phosphorus (gravimetric method), according to the analysis submitted by the supplier.

Note: Because lipids are comparatively difficult to digest, perchloric acid often has been used for this purpose, despite the necessity of taking proper precautions to avoid explosive conditions (1, 2). In the above procedure, the initial oxidation steps should be carried out behind a safety-glass shield in a fume hood and the analyst should wear protective rubber gloves and a face-shield.

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A CURD FIRMNESS TEST FOR COTTAGE CHEESE[1]

An objective test to measure the firmness of cottage cheese curd is needed for research and manufacturing control. The Lundstedt cheese curd meter (2), which requires about 400 g. of curd for testing, is especially useful as a control in the manufacturing process, but lacks sensitivity when applied to finished curd.

The purpose of this paper is to describe a test for curd firmness that uses only a small amount of curd, is sensitive enough for use on finished curd, and is simple enough to use for control purposes during manufacturing.

Equipment and method of performing the curd firmness test. The apparatus for measuring curd firmness is shown in Figure 1. It is a modification of the Cherry-Burrell curd-tension meter (1). The slotted container holds the curd into which the wire cutter is forced by the driving mechanism. The resistance offered by the curd is registered in grams on the 500-g. dietetic scale.

The curd container is a slotted cylinder made from 2-in. stainless steel piping which is closed at the bottom by soldering to it a circular, flat piece of stainless steel. A 5-lb. weight for pressing the curd into the cylindrical container, and the slotted container itself, are shown in Figure 2. The weight is solid stainless steel machined to fit snugly, yet to slide freely into the container when it is placed on top of the curd.

The wire cutter is a U-shaped frame with a 3-in. length of 0.016-in. stainless steel wire stretched tautly from tip-to-tip of the U-frame. The base of the U is joined by a vertical bolt to the driving mechanism. The base of the U is extended to form an arm which moves smoothly and securely in a vertical guiding slot on the fixed frame of the driving mechanism; this guide prevents the wire cutter from twisting and keeps the wire from touching the slotted container when force is applied.

The driving mechanism forces the wire cutter downward at a rate of 2 in. in 15 sec.

The test is made by packing curd into the cylindrical slotted container with a spoon. It is packed firmly with the spoon, after each spoonful is added, to minimize pockets of air or liquid between curd particles. When the container is full, the 5-lb. weight is placed on the curd and balanced there, by hand if necessary, for approximately 10 sec., to pack the curd uniformly. The slots in the container must be closed during this pressing interval, to prevent the escape of curd; two flat pieces of stainless steel held lightly against the slots serve this purpose. At the same time, free whey or cream may escape without loss of curd.

The container of curd is then positioned on the dietetic scale under the wire cutter so that the cutter can be forced downward into the curd without touching the sides of the slotted container. Irregularities of consistency in the packed curd cause some fluctuations of

[1] Approved for publication by the Director of the Wisconsin Agricultural Experiment Station.

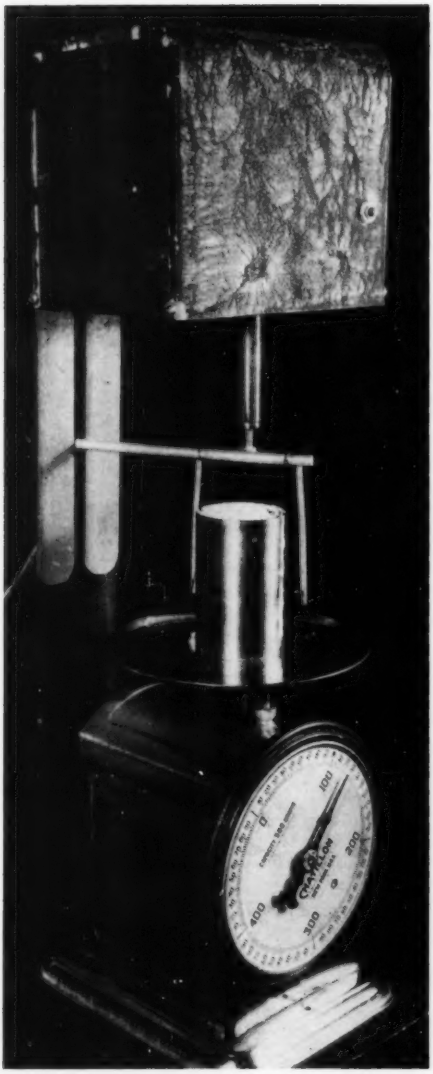

FIG. 1. Apparatus for making the curd firmness test, showing the driving mechanism, the wire cutter, the curd container, and the dietectic scale.

pressure as the wire is forced through the packed curd. An average of the highest value and the lowest value, observed as the wire is passing through the curd, is taken as the measure of firmness in grams.

All measurements in this study were made in a 50° F. walk-in refrigerator, using large-curd cottage cheese which had been cut with ½-in. knives.

Evaluation of the curd firmness test. Recording readings. A study was made of two methods of recording firmness readings during the passage of the wire through the curd. In Method I, only the highest reading was recorded. In Method II, an average was taken of the lowest and highest readings observed during the passage of the wire through the curd. In this comparison, the highest readings in Methods I and II were the same.

Table 1 is based on a total of 182 measurements on 25 samples of uncreamed salted curd, and shows the average firmness, the pooled standard deviations, and the pooled coefficients

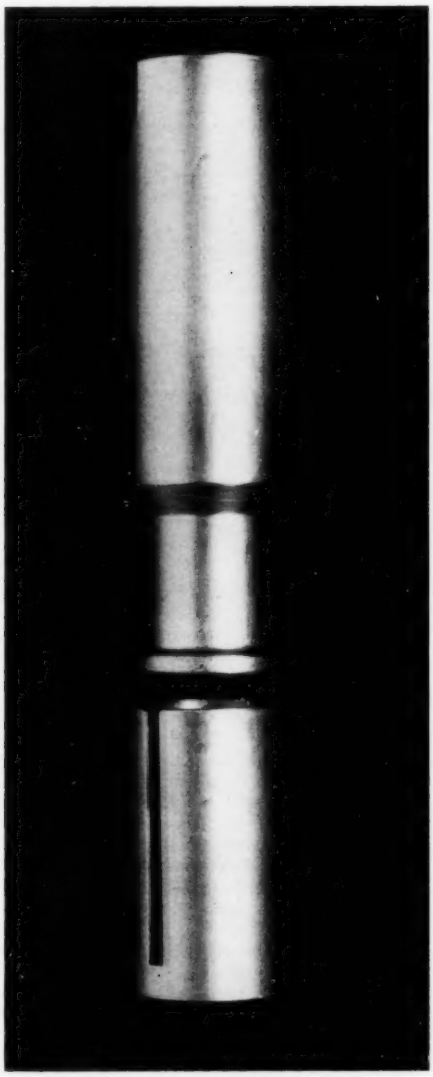

FIG. 2. The slotted container and the weight used for pressing the curd into it.

TABLE 1

Comparison of two methods of recording curd firmness values observed on the scale during passage of the wire through the curd

Observation	Method I[b]	Method II[c]
Average firmness[a] (g.)	257.8	220.1
Pooled standard deviation (g.)	±20.12	±13.47
Pooled coefficient of variation (%)	± 7.80	± 6.15

[a] Average firmness of 182 observations on 25 samples of salted, uncreamed curd. Six or ten replicate determinations were made on each sample.

[b] The highest reading observed on the scale as the wire passed through the curd.

[c] The average of the highest reading (same as that in Method I) and the lowest reading observed on the scale as the wire passed through the curd.

of variation obtained by each of these two methods. Method II was selected because its coefficient of variation, 6.15%, was lower than the 7.80% obtained by Method I.

Precision. The precision of the firmness test was estimated from replicate determinations of curd firmness on a total of 105 samples of creamed curd and 30 samples of uncreamed curd. Each sample was classified into one of four groups, according to the magnitude of the mean value for its measurements of firmness. The pooled values for standard deviations and coefficients of variation of each group are shown in Table 2, for creamed and uncreamed curd.

Coefficients of variation were relatively constant from one class of firmness to the next, for both creamed and uncreamed curd; they tended to be slightly higher for the creamed than for the uncreamed curd. The precision, as indicated by the pooled standard deviations within each class of firmness, decreased as the firmness of the curd increased.

The variability that is shown in Table 2 can be attributed, in part, to the method itself, and in part to the inherent irregularity of firmness between particles within each sample.

Relationship between the curd firmness test and organoleptic observations of the curd firmness. An experiment is reported here to show the degree of correlation between sensory observations of firmness and the curd-firmness test. Four vats of cottage cheese with different degrees of firmness, ranging from soft to hard, were made on each of two consecutive days. Curd made on the first day was 36 hr. old at creaming, whereas that made on the second day was 14 hr. old. Cream containing 12% fat was added to part of the curd from each vat to give 4.5% fat in the creamed curd. Samples were stored at 50° F. and organoleptic and machine observations of curd firmness were made at that temperature 24 hr. after creaming. All samples were prepared in duplicate, coded, and presented in random order to four judges for organoleptic evaluation of firmness. The judges were asked to grade the cheese as "too firm," "firm," "desirable," "soft," or "too soft." After scoring, these descriptive grades were assigned values of 5, 4, 3, 2, and 1, respectively, for the analysis of the data. Since the four judges scored each of the duplicate samples, each organoleptic value for firmness for each sample represented the average of eight judgments. The curd firmness test was made in quadruplicate on each sample.

Figure 3 shows the trends of relationship between the sensory observations of firmness and the curd-firmness test for creamed and uncreamed cottage cheese. A curd firmness of about 120 g. appears to be desirable for creamed curd and of about 170 g. for uncreamed salted curd, according to the lines of relationship drawn by the method of least-squares. Coefficients of correlation between the organoleptic observations and the curd firmness test for

TABLE 2

Pooled standard deviations and coefficients of variation for curd firmness measurements within different ranges of firmness for salted, uncreamed curd, and creamed curd

Classification of mean values of curd firmness	Number of lots of curd in each group	Average firmness of curd in each group	Total number of observations in each group	Pooled standard deviations by groups	Pooled coefficient of variations by groups
(g.)		*(g.)*		*(g.)*	*(%)*
		Uncreamed curd			
99 or less	7	86.2	42	± 3.47	±4.02
100–149	12	116.8	68	± 5.11	±4.38
150–199	7	170.4	40	± 6.30	±3.70
200 or greater	4	226.0	20	± 6.94	±3.07
		Creamed curd			
49 or less	8	39.3	48	± 2.61	±6.65
50–59	57	74.5	272	± 4.86	±6.52
100–149	30	117.5	170	± 6.25	±5.32
150 or more	10	173.3	54	±12.10	±6.98

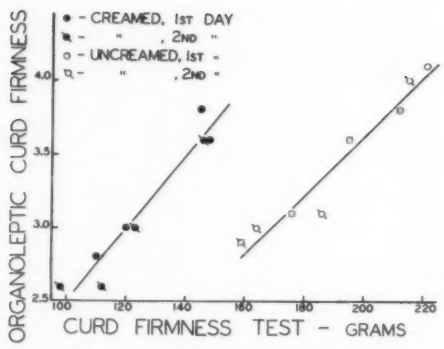

FIG. 3. Relation between organoleptic observations of firmness and the curd firmness test for creamed and uncreamed curd made on two consecutive days.

the creamed and uncreamed curd were .96 and .97, respectively.

A corollary to these observations is that the uncreamed curd from each vat generally scored higher on the scale of firmness than the same curd after creaming. Curd that was satisfactory as uncreamed curd was judged to be slightly soft as creamed curd. Curd that was of satisfactory firmness as creamed curd was considered slightly firm as uncreamed curd.

Preliminary results of a survey of cottage cheese distributed commercially in Wisconsin indicate that the test for firmness, as described in this paper, is less reliable in predicting organoleptic firmness of creamed small-curd cottage cheese than it is for the large-curd style. This survey, still in progress, is being made in cooperation with the Wisconsin Department of Agriculture.

Relationship between total solids and curd firmness. The total solids of curd before salting and the curd firmness values of the salted, uncreamed curd were compared in 27 lots of curd. These lots of curd were manufactured for other experiments and included differences caused by such variables as methods of washing the curd with water, cooking temperatures, and cutting acidities. Statistical analysis of the data showed a highly significant correlation ($r = .78$) between the curd firmness and the total solids with a regression equation of

$$Y \text{ (curd firmness)} = -301.9 + 21.95\,X \text{ (per cent total solids)}$$

However, quite wide confidence limits for individual observations were obtained, indicating curd firmness to be an inaccurate measure of the total solids in the curd. Resolution of the factors involved in the relation between curd firmness and total solids may make curd firmness measurements a more accurate measure of total solids under a standardized condition of manufacturing.

SUMMARY AND CONCLUSIONS

Equipment and a method for measuring the firmness of large-curd cottage cheese have been described. A close relation was established between this method and the organoleptic evaluation of curd. Under the conditions of these experiments, firmness of uncreamed curd before salting could not be used to predict accurately the total solids content of the uncreamed curd after salting.

This method of measuring curd firmness should prove useful wherever an objective evaluation of curd firmness is needed in experimental research or in the commercial laboratory.

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SYMPOSIUM: LATEST DEVELOPMENTS IN THE HEAT AND VACUUM TREATMENT OF MILK[1]

EQUIPMENT FOR VACUUM TREATMENT OF MILK[2]

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The principle of vacuum treatment of milk and milk products is not new, but some of the equipment is new. One manufacturer has had a vacuum pasteurizer available for many years which was developed especially for cream. Another major company has had a vat pasteurizer for many years for maintaining a vacuum of about 10 in. Hg. The equipment for continuous vacuum treatment, in combination with separate short-time pasteurization equipment, has been developed recently. The first papers in this country on this subject were written within the last three years by Roahen and Mitten (5), Barber (1), Ehrman (3), and Roberts (6).

EQUIPMENT AVAILABLE

1. Combination vacuum and pasteurization equipment in a single unit for continuous treatment has been available about 20 yr. Behlmer (2) and Tracy (7) have published recent articles on the equipment, which uses steam injection.

2. Batch pasteurization with a vacuum which may be applied during heating, holding, and cooling processes.

3. Equipment for vacuum treatment during pasteurization, which may be a part of a high-temperature short-time (HTST) or ultra-high temperature (UHT) system, in separate or remote container or containers, and is described herein.

Remote continuous flow vacuum treatment equipment. The vacuum equipment in this category may be classified as one-chamber or two-chamber, or by the method of product treatment such as steam injection, air scrubbing, rapid agitation through venturi (strictly not vacuum treatment but vapor pressure phenomena), or cooling of the product. The effectiveness of removal of weed and feed flavors is reported by Roberts (6) to be related to the quantity of steam used. The codes in some areas do not permit live steam injection; therefore, equipment has been developed to accomplish vacuum treatment where these restrictions are in effect. Some units can be operated either with or without steam injection, according to Roahen and Mitten (5). Several methods of connecting the unit to the HTST equipment are used (Figure 1). Generally, the milk is taken from the short-time system after the raw milk is heated at the regenerator, the heating section, or after the flow diversion valve.

Temperature-heat-vacuum relationships. The relationship of the temperature of the milk entering the chamber and the vacuum of the chamber determines whether the product will cool as it enters the chamber. If milk at 170° F. (Figure 2) is placed in a chamber at a vacuum of 20 in Hg, the product will cool to 161° F. The heat will evaporate moisture until the product is cooled. A product condition specified by a point to the right of the curve would cool as it evaporates water to the condition described by the curve.

The product is heated to a temperature above the boiling point of some of the constituents, causing most of the undesirable odors and flavors to be evaporated and removed. At 161° F. the latent heat is 1,000 B.t.u. per lb. of water evaporated (Table 1). Condensation or dilution of the product can be avoided by proper control.

TABLE 1

Temp. °F.	Vacuum, in. Hg	Absolute pressure, p.s.i.	Latent heat, B.t.u/lb	Enthalpy, (total heat) B.t.u/lb
100	28.0	0.95	1,037	1,105
120	26.5	1.7	1,026	1,114
140	24.1	2.9	1,014	1,122
160	20.3	4.7	1,002	1,130
180	14.7	7.5	990	1,138
200	6.5	11.5	978	1,146
212	0	14.7	970	1,150

HEAT FLOW

As the product moves into the vacuum unit, it comes under the influence of the pressure and temperature changes. Heat may be added by direct injection of steam or by indirect means. Heat may be removed by evaporation and removal of volatiles and water vapor; heat may be lost by the product to the walls of the vacuum treatment unit, either to cooling water or to the room.

The following example illustrates the temperature changes in the product: A two-vacuum chamber using steam injection is described. The flow is as follows: 10,000 lb. per hour of milk at 162° F. flows from the HTST unit to the first vacuum chamber at 9.5 in Hg. Steam is added

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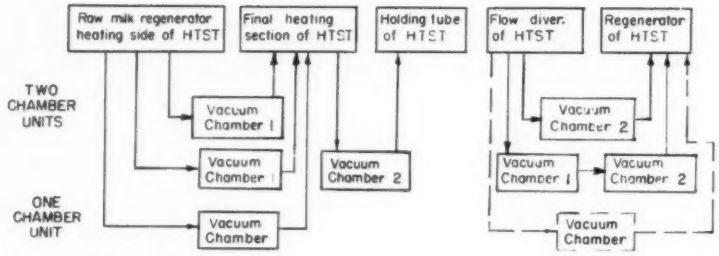

FIG. 1. Methods of attaching vacuum treatment equipment to HTST

at the rate of about 10 BHP, which is equivalent to 334,650 B.t.u. per hour or 341 lb. per hour of steam. The steam would heat the milk 34.1° F. if there were no heat losses or vaporization. With a vacuum of 9.5 in Hg, evaporization will take place at 194° F. and the temperature will not go above 194° F. If there were no heat loss and no vacuum, the product would heat to 196.1° F. The calculated heat loss from the first chamber of a particular unit is 3,983 B.t.u. per hour (Figure 3), which would be equivalent to reducing the temperature at full flow, 0.40° F. Considering the calculated heat loss without vacuum, the product would heat to 195.7° F.

The product then goes to the second chamber, which is under a higher vacuum (lower pressure) of 22 in Hg, where it is cooled to 152° F. by evaporation of the moisture and other constituents. The calculated heat loss from the second chamber at 152° F. is 3,400 B.t.u. per hour, which is equivalent to a temperature drop of 0.35° F. To cool 10,000 lb. per hour of product and 316 lb. per hour of water used for heating from 194 to 152° F. would give up 406,000 plus 13,270 or 419,270 B.t.u. of heat, and would evaporate 390 lb. of water or equivalent, which essentially would be the steam added in the first chamber. The difference in steam quantity is due to heat losses of chamber and piping, heat required to vaporize volatile components, and heat for removal of air from the product. Data on heat requirements to remove air and to volatize and remove other condensables and noncondensables are not available. The total calculated heat loss from the two chambers accounts for a temperature drop of only 0.75° F. for full-capacity flow. With a lower flow rate, the temperature drop would be greater.

CONTROL

Proper control of steam flow, milk flow, and vacuum is required to avoid concentration or dilution of the product. The ratio of steam con-

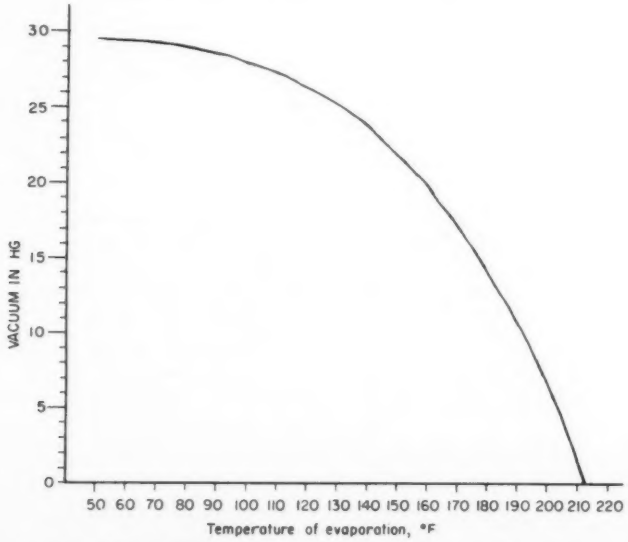

FIG. 2. Effect of vacuum on evaporation temperature of water.

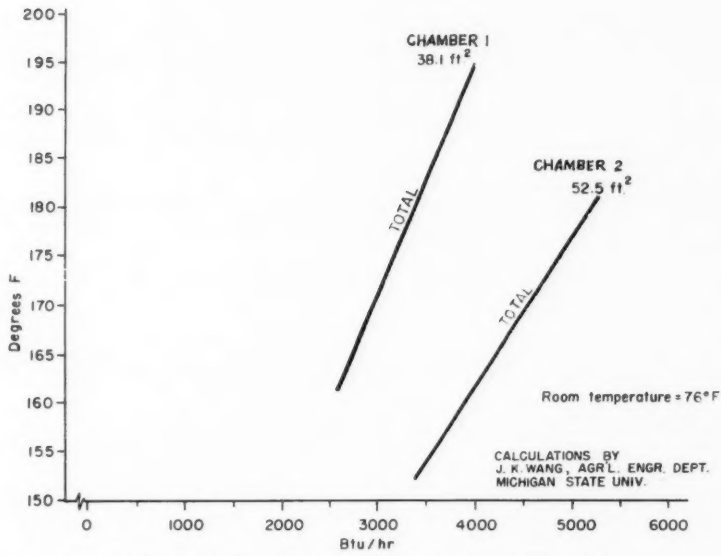

FIG. 3. Calculated heat loss from surface of vacuum chambers.

densed in the first chamber to water vaporized on the second chamber for a unit with steam heating, determines the change in concentration of the product. To avoid change in concentration, a 1:1 ratio is desired. With no heat losses, equal addition and removal of water will be obtained if the temperature increase in the first chamber is equal to the temperature decrease in the second chamber. From the preceding example, it can be noted that it is necessary to have a lower outlet temperature than inlet temperature, to provide heat for vaporization and removal of components and to replenish heat losses. As the vacuum is decreased (pressure increased), the temperature of evaporation is increased. To remove more water in the second chamber, the temperature of evaporation is decreased by increasing the vacuum. An automatic control device is used for sensing the temperature on the inlet and outlet of the vacuum unit. The control can be set to maintain the desired temperature by regulating the vacuum by admitting more or less atmospheric air. The outlet is usually maintained about 3 to 5° F. below the inlet temperature, to maintain the density.

VACUUM CHAMBER DESIGN

Steam heated. Milk droplets are dispersed in the top of the unit and steam usually into the bottom of the unit, to heat the milk to or near the temperature of evaporation corresponding to the vacuum maintained. In the second chamber, material with a boiling point above that corresponding to the evaporation temperature at the vacuum maintained will be removed with the heat available in the product. With some units, volatiles are removed in the first chamber, as well. Also, steam may be placed in the milk under pressure ahead of the vacuum chamber and then additional steam added at the vacuum chamber.

Without steam. Filtered air may be bubbled through the product to release the volatile materials. One- or two-chamber units may be used.

One commercial unit does not use steam or vacuum but operates on the principle of difference in vapor pressure. The product at 162° F. enters a large cylinder and is forced through a venturi, around which additional product is picked up and a thin film sprayed inside. The noncondensable gases rise and are carried from the system by a cold-water spray. The product may be circulated several times through the venturi before it is removed from the system.

In one commercial unit, the milk is flashed from the raw milk regenerator at 135° F. into a cylinder maintained at a vacuum of 25 in Hg. The walls of the unit are cooled by city water, so as to completely recondense the vapor, so that the only loss or material removed from the system are the noncondensable volatiles. The cooling provides a temperature drop of approximately 4–5° F.

REMOVAL OF CONDENSABLE AND NONCONDENSABLE GASES

Both condensable and noncondensable gases must be removed from a unit heated with steam. Units using filtered air or other means of removing noncondensables are designed principally for removal of noncondensables rather

than condensables. Approximately one gal. per minute of water heated 80° F. above inlet temperature is used for a steam-heated unit for each 1,000 lb. per hour of product capacity. Those for removing noncondensables require about 1 gal. per minute for each 8,000 to 10,000 lb. per hour capacity.

The vapors may be removed by *(1)* condensing on a cool heat exchanger operated in conjunction with a vacuum pump, or *(2)* an ejector condenser. Units using steam injection normally use the former method. If only noncondensables and low-temperature volatiles are removed from the final chamber, the latter method is often used.

SUMMARY

Several types of equipment are available for vacuum treatment of milk and milk products used at the time of pasteurization. Units of the remote type are constructed as one- or two-chamber and may not use steam injection. By proper control with available equipment, dilution and concentration can be avoided. For units using steam injection, the outlet temperature is maintained about 3–5° F. below the inlet temperature to avoid change in density. The lower outlet temperature is needed to supply heat to make up losses from heat transfer, heat required for removal of air, heat for evaporation of low-temperature volatiles, and for the removal of noncondensables. Air scrubbing, cooling heated milk, and rapid agitation through a venturi are other means of removing undesirable odors and flavors.

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PROBLEMS INVOLVED IN FLAVOR REMOVAL

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Relatively recent reviews of off-flavors in milk have been made by Strobel, Bryan, and Babcock (26), McDowall (10), and Hunziker (7). A large number of weeds, plus many important feedstuffs, are reported by these authors as being responsible for objectionable flavors in milk and dairy products. The ability to use these feedstuffs to maximum advantage nutritionally and economically would be of tremendous benefit to the dairy industry.

CHEMICAL NATURE OF TAINTING SUBSTANCES

Little is known pertaining to the flavoring substances in milk. Aurand (2) separated four fractions of flavoring compounds by steam distillation from a mixture of wild garlic (*Allium vineale*) and wild onions (*Allium canadense*). These fractions were collected at 32–40° C., 65–70° C., 85–90° C., and at 100° C. The two low-temperature fractions composed approximately 15% of the distillate and the remainder (about 80%) was obtained at the higher temperatures of fractionation. Mass spectrometric analysis showed that at least 12 different chemical substances were present and that they were composed primarily of di-sulfides and mercaptans. Several gaseous emanation products of the edible onion were reported by Niegisch and Stahl (17). Some of these were propyl mercaptan, propionaldehyde, acetaldehyde, carbon dioxide, methyl alcohol, propyl alcohol, sulphur dioxide, hydrogen sulfide, and di(n-propyl) sulfide.

Some feeds or weeds that give an off-flavor to milk when they are consumed by the cow along with the suspected chemical(s) are given below. Without doubt, as better methods of analysis are used, it will be found that a combination of compounds is responsible for the typical off-flavors associated with many feeds and weeds.

Generally, it is believed that most of the substances that are responsible for off-flavors are more soluble in butterfat than in water or the skimmilk portion. However, the amount of most of the substances required to produce a distinct flavor is in the range of parts per million. Therefore, the relative solubility may not be too important in considering methods of volatilizing the various substances.

Other complications exist in the isolation and identification of the chemical substances. The most abundant sources of the substances are the plants. Oftentimes, there appears to be a close relation between the odor of the plant and that found in the milk. However, in many instances,

Name	Botanical classification	Chemical	B.P.* °C.	Solubility* in water
Land cress (10)	*Coronopus didymus* or *Senebiera didyma*	Benzyl mercaptan	194-5	i.w.
Penny cress (10), stinkweed, or French weed	*Thlaspe arvense*	Allyl isothiocyanate	150.7	0.2w.
Penny royal (10)	*Mentha pulegium*	Pulegone	224	i.w.
Pepper grass (10)	*Lepidium virginicum*	Indole	254	s.h.w.
Onion and garlic (2, 17)		Di-n-propyl sulfide	141-2	i.w.
		Isopropyl mercaptan	60	sl. s.w.
		Propionaldehyde	48.8	20^{20} w.

* *Handbook of Chemistry and Physics.* 23rd ed., Chemical Rubber Publ. Co. 1939.

the extracts from the plant do not produce the same flavor when added to milk that occurs from feeding it. This suggests that some of the flavor-producing substances undergo change in the digestive processes of the cow, or substances may be produced by fermentation and digestion that result in objectionable flavors.

Another factor that perhaps needs mentioning is that little consideration has been given to the possibilities of chemical changes in the substances during processing for removal. Subjection to relatively high temperatures could cause a decomposition of the substances or a reaction with the milk constituents. If the end products were flavorless, this would be good. On the other hand if, as a result of interactions, they give rise to off-flavors, the problem of flavor removal would only be aggravated. Scott (23) reported that the taint originating from land cress does appear to form from another substance at the temperature of pasteurization, and that a definite time lag is necessary to complete the reaction. This makes it impossible to render the cream free of this taint.

The lack of knowledge about the chemical nature of these flavor-producing substances complicates the problem of evaluating the means of removing them from milk and dairy products. The senses of taste and smell are valuable tools for testing for chemical substances, but they often leave something to be desired in terms of objectivity. Also, some of the treatments produce chemical changes that may cover up or mask flavors rather than removing them.

METHODS OF REMOVING TAINTING SUBSTANCES

Taints in milk and dairy products have been removed partially or wholly by (1) dilution or washing with water or a light weight mineral oil (8, 9), (2) aeration with and without reduced pressure (7), (3) application of vacuum, and (4) distillation with steam usually under controlled pressure. Since methods (1) and (2) have not proved satisfactory for reasons of legality and inefficiency, they will not be discussed.

It has been known generally for several years that vacuum treatment alone would bring about some improvement in the flavor of dairy products. However, it has also been recognized that vacuum treatment was not sufficient to remove all of the flavoring substances that are in milk under normal spring grazing conditions. The failure to remove all of the flavoring substances may be readily understood when one realizes that the usual vacuum applied for flavor removal is seldom above 26 in. (approximately 100 mm.) and that there are many flavoring substances with a vapor pressure lower than this at processing temperatures. The primary functions of vacuum are to accelerate the removal of flavor-laden vapors and to assist in temperature control.

The benefits of steam as a distilling agent have been demonstrated by several investigators (3-7, 10-15, 18-25, 27, 18). They favor steam because (1) some substances are very steam-volatile, (2) the temperature of the milk or dairy product can be controlled by steam during processing, (3) steam is readily available, and (4) the condensate can be disposed of easily and quickly.

In discussing the practical applications of steam distillation, Adams and Johnson (1) stated that the substance to be steam-distilled must be insoluble or only sparingly soluble in water; prolonged contact with boiling water or steam must not decompose it; and the vapor pressure must be at least 5 mm. in the neighborhood of 100° C. McDowall (13) studied the factors that affect the rate of removal of substances from cream. He found that the rate of removal of a dissolved tainting substance from cream by steam distillation depends upon the relative volatility of the pure substances, the temperature of steam distillation, and the influence of water on the vapor pressure of the tainting substance in dilute solutions. He pointed out that the boiling point of a substance

under atmospheric pressure could not be taken as a reliable index of its volatility in steam. For example, butyric acid (b.p. 163° C.). is more steam-volatile than formic acid (b.p. 101° C.). When the effect of the change in pressure on the ratio of the vapor pressures of several organic substances to the vapor pressure of water was compared over a wide range in pressure (0–800 mm.), he stated, "we may conclude that for ideal solutions lowering the temperature and pressure, i.e., increasing the 'vacuum applied': (1) would facilitate the removal of low-boiling substances; (2) may facilitate or may retard the removal of substances with boiling points close to that of water; and (3) would definitely retard the removal of high-boiling substances."

While there is much work to be done in isolating, identifying, and studying the chemical behavior of the tainting substances found in milk and dairy products, the experience of the New Zealand workers and others indicates that most substances are removed at high temperatures and low vacuums.

Steam is readily available in most instances, but its wise use is sound economies. The amount of steam required for treatment of dairy products is determined by the taint that is most difficult to remove, and the efficiency by which the escaping steam vapors can be saturated with the taint vapors. The extent to which the steam can be saturated with taint vapors is dependent upon time, i.e., "the required time of contact for a given degree of taint-removal is approximately inversely proportional to the square of the diffusion rate" (23), and the manner whereby the steam is created or injected. Scott (23) used diacteyl (b.v. 88° C.) and acetoin (acetyl methyl carbinol, b.p. 150° F.) to compare the relative effectiveness of open steam (conditions where the rate of steam entering the system is equal to that leaving it) and flash steam (steam that is boiled from the liquid) for taint removal. He found that flash steam was about as effective in removing the more volatile taints as open steam; however, flash steam was much less effective than open steam in reducing the less volatile taints. Further (24) work with multiple contact systems, counterflow arrangements, and multiple contact counterflow methods demonstrated more effective and efficient means of taint removal. The theoretical and applied research of Scott and McDowall is of great value in giving direction to the solution of our taint-removal problems with fluid milk and related products.

QUALITY OF STEAM

One of the most serious objections raised to the use of steam for removal of tainting substances is that the dairy product will be contaminated. Some water supplies require extensive treatment with chemicals prior to their use in boilers. Since most steam supplies have been used for heating, little attention was given to the properties of the chemicals, such as toxicity and odors or flavors. The primary concern was to keep the boiler and steam lines in good condition. An entirely different quality of steam is necessary for direct injection into food products. It must be free of rust, relatively dry and low in superheat, and free from any compounds that will affect flavor or endanger health.

Reliable manufacturers of compounds for boiler treatment can prescribe and furnish compounds that are satisfactory for use when direct steam injection of food products is practiced. Generally, compounds that contain lignins and tannins must be omitted or refined so that these substances are eliminated. Excessive chlorine in the water supply may result in a medicinal flavor in the finished product.

Some manufacturers have been recommending certain volatile ammonium compounds in boilers to reduce the corrosion in the steam lines. The ammonium volatilizes and then neutralizes the acid formed in the lines, thus reducing the corrosion. Some of these compounds are toxic and their use has been declared illegal by The Federal Food and Drug Administration. The elimination of these compounds from most dairy plant steam supplies does not seem to be a serious problem. The rust, wetness, and superheat can be controlled by the use of strainers or purifiers and by placing the reducing valves a sufficient distance from the processing equipment so that the superheat is dissipated before the steam contacts the dairy product.

Generally, it appears that most dairy plants can have an acceptable steam supply from commercial boilers, provided that they are operated properly, recommended water treatment practices are followed, and the installation is properly engineered.

PRESENT STATUS

Vacuum and steam distillation treatments have been used for cream and ice cream mix in North America for many years (3–7, 18, 22, 27, 28). The process was approved for use on fluid milk in the United States in 1954. Since then, more information has become available on its applicability to fluid milk products (16, 19, 20, 21, 29).

There are many problems inherent in the use of taint-removal equipment for fluid milk products that are not encountered with cream for buttermaking and for ice cream mix. Usually, the intensity of taint is not so great in milk for fluid consumption. Dilution or concentration must be controlled. Sanitation standards are higher. The entire product is consumed, so there is no opportunity to remove some substances in the buttermilk as in the buttermaking process. Some of the products such as creamline milk are not readily adapted to the new processes.

Nevertheless, there are many kinds of flavor-removal equipment on the market. It is noteworthy that most of the users are satisfied with the results that are being obtained. The effectiveness of the machines under commercial con-

ditions has been partially evaluated. The results indicate that some steam treatment is necessary to get the degree of flavor removal desired by many dairy plants. However, others report favorable results with vacuum treatment plus occasional flash steam. Controls have been developed that will prevent concentration and dilution. Fluid cream and chocolate milk have been processed through some of the equipment with good results.

Excellent progress has been made, but there are still problems that must be overcome. Some of these are: What is the chemical nature of the tainting substances? What is the particular combination of time, temperature, vacuum, and/or steam treatment that is most effective and efficient? Can tests be developed for the tainting substances, so that more objective means will be available for evaluating the treatment combinations? Can the treatments be adapted to enough of the dairy products to insure greater economy in the use of the equipment? Can the equipment be adapted to remove chemical substances that are not affected by present treatments? Obviously, the answer to these questions is "yes," but much work is still to be done before maximum advantage can be taken of the potentials that are available.

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BACTERIOLOGICAL ASPECTS [1]

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It has been emphasized in the two previous papers that the trend toward high heat and vacuum treatment of milk offers many advantages in the production of improved milk products. These treatments result in a product of improved keeping quality and often a vast improvement of flavor. The physical-chemical and the engineering aspects of these treatments have been discussed. This discussion is concerned with the bacteriological aspects of heat and vacuum treatments of milk.

Consideration of the bacteriological aspects of the heat and vacuum treatment of milk must be based on an evaluation of the severity of the heat treatment, sanitary design of the equipment, and handling of the finished product. The vacuum treatment involved in these processes removes off-odors and flavors to varying degrees, but has little bactericidal significance. Bacterial destruction is obtained, for the most part, by the heat treatment provided by the unit itself, or by conventional high-temperature short-time (HTST) units operated in conjunction with the vacuum treatment. In fact, practically all of these units are operated in conjunction with regular pasteurization equipment.

A study of the various types of equipment available for the heat and vacuum processing of milk discloses that the milk is subjected to 180 to 190° F., in addition to conventional pasteurization. Some equipment is so designed that the unit can be used as a pasteurizer. In this instance, a temperature of 194° F. is maintained in the first chamber. It can be seen, then, that under ordinary operating conditions the milk is subjected to heat that is more than adequate for proper pasteurization. However, regardless of how obvious the adequacy of the heating might appear, controlled bacteriological studies always are necessary.

It has been recognized for many years that the most satisfactory procedure to use in the evaluation of new types of equipment for the processing of milk is the inoculation of the product with known numbers and types of organisms, followed by the determination of the destruction of them by the heat treatment. As far as could be determined, only one type of equipment which utilized these heat and vacuum treatments had been evaluated by this particular procedure.

In spite of the fact that the studies just mentioned were not only carried out a number of years ago—and, therefore, can not be classed as a late development—but were designed specifically to investigate HTST pasteurization of ice cream mix, it is believed that mention should be made of this earlier work. Three groups of investigators, Speck *et al.* (7), Tracy *et al.* (9), and Barber and Hodes (1), utilized the techniques of the addition of heat resistant test organisms of various types to ice cream mix and subjecting this inoculated mix to the heat and vacuum treatments of pasteurization.

All of these workers showed that heating at 190–200° F. for very short holding times resulted in bacterial destruction equivalent to or better than that obtained by the conventional holding method of pasteurization. Trials were also made in which the destruction of the normal flora was investigated. Here, also, the bacterial destruction was satisfactory, although frequently spore formers made interpretation of results difficult. In fact, as a result of Speck's studies with the Vacreator, the United States Public Health Service a few years ago approved the use of this equipment for the pasteurization of dairy products, when operated so that the temperature in the first chamber was maintained at 194° F.

Since December, 1957, attempts have been made to locate areas where systematic bacteriological studies might be in progress on these heat and vacuum treatments of milk. Many universities were contacted, but in every case no late information appeared to be available. However, through the kindness of Tittsler and Hanrahan (8) of the Bureau of Dairy Industry, some very recent bacteriological data have been made available for this discussion, on the process these investigators are studying.

The Hanrahan steam injection pasteurization process consists of forewarming milk to a given temperature, injecting steam to a higher temperature followed by flashing into a vacuum where the product is cooled to the original forewarming temperature and, finally, cooling to refrigeration temperatures. The process takes 15 sec. for heating from 40–50° F. to the forewarming temperatures; 15 sec. for steam injection to the higher temperature, and 10 sec. for vacuum cooling to the forewarming temperature. The process is quite rapid.

Tittsler's (8) bacteriological data on five trials are summarized in Table 1.

These studies were made using raw milk without the addition of any test organism other than the normal flora. The per cent destruction obtained by this process ranged from 97.5 to 99.98. In the two trials where a conventional HTST pasteurization was used as a control, the destruction was 99.56 vs. 99.62 and 97.5 vs.

[1] Due to unforeseen difficulties, this paper, as such, was not presented at the 1958 annual meeting. The subject was discussed ably by R. P. Tittsler, M. L. Speck, and F. E. Nelson. The author wishes to express his thanks and appreciation to them for their assistance on very short notice.

TABLE 1

Trial	Starting temperature	Forewarming temperature	Steam injection temperature	Vacuum temperature	Raw count	Pasteurization count	Test tube 85° C. 15 sec. count
	(° F.)				(ml.)		
III	45–50	135	165	135	525,000	190	40
	45–50	145	165	145	525,000	170	
	45–50	155	165	155	525,000	130	
IV	45–50	138	161	138	138,000	80	70
	45–50	145	161	145	138,000	170	
	45–50	155	167	155	138,000	270	
V	45–50	145	163–164	145	225,000	1,000	450
		(Conventional pasteurization, 163–164° F. 15 sec.)					850
I	45–50	about 155	165–170	155	61,000	5	
II	45–50	.....	165–170		4,800	120	70
		(Conventional pasteurization, 163–164° F. 15 sec.)					70

98.54, for experimental vs. control, respectively. These results indicate that this particular process resulted in a satisfactory reduction of bacteria. It is evident also that the steam injection process gives results that are comparable to those obtained in the studies previously mentioned, using the Vacreator and heat resistant test organisms and normal flora.

There are a number of investigators who are concerned with the new high heat treatments of dairy products. These treatments are aimed toward the production of a sterilized product or at least a product with prolonged keeping quality. Bacteriologically, the problem in these processes is the variability in the raw milk flora. When spore forming types are present, the goal of sterility is more difficult to achieve.

Kaufman (3) has provided data on his studies with the Roswell Heater and what he calls ultra high temperatures (UHT). Milk processed in this unit at 206° F. for 3 sec. gave a count of less than 100 per milliliter. After 18 days, the count increased to 3,000 per milliliter. The same milk processed at 176° F. for 16 sec. gave a one-day count of 20,000/ml, which increased to 87 million/ml on the 16th day. Kaufmann (3) states that the lag phase for surviving organisms in the UHT treatment is 15 days, as compared to from two to three days in the conventional HTST process.

The Michigan workers also report that higher temperatures presented no problem. Milk processed at 272° F. was held at room temperature (70° F.) for ten days and still had an acceptable flavor. In another experiment, where the processing was 232° F. for 1 sec., the product had a count of less than 100/ml after 46 days at 40° F. Kaufmann comments that these results indicate what can be done, but cautions that there is need for more work.

Frazer (2) has reported on his studies in the production of a sterile concentrate. The product is subjected to temperatures during processing of 285° F. He reports that the keeping quality of this product depends upon the numbers and kinds of organisms surviving processing or entering the product after processing. When these studies were reported in March, 1958, it was stated that a sterile product is possible and that plans for future work included the use of inoculation of the product with spores, to check further the bactericidal efficiency of the process.

It has been noted that the effectiveness of these new high heat treatments depends upon the numbers and types of organisms present in the starting milk. With this fact in mind, a few comments might be made regarding the heat resistance of various types of organisms which might be present in the raw milk supply.

Olson and coworkers (5) have studied the heat resistance of psychrophiles and heat resistant micrococci. As far as the psychrophilic organisms are concerned, conventional HTST pasteurization has been adequate for the destruction of these organisms. There do not appear to be any so-called psychrophilic strains with heat resistance which would cause problems when processing temperatures are high. With these organisms post-pasteurization contamination is a far greater problem and care must be taken that the processed product does not become contaminated even with very low numbers of psychrophiles.

Myhr and Olson (4) investigated 39 strains of micrococci and found 13 cultures survived 143° F. for 30 min. However, thermal death time curves indicated that at the temperatures used in the new high heat treatments the micrococci should cause no problem.

When new methods of pasteurization or the heat treatment of milk are considered, the investigator is faced with the problem as to whether the heat treatment in question is adequate for the destruction of pathogens. Litsky and coworkers (6) have been investigating the destruction of various pathogens by heat treatments, using very short holding times. These

workers have used a 0.25-sec. holding time with a come-up time of 0.05 sec., and a 0.5-sec. holding time with the same come-up time of 0.05 sec. Their results show the following temperatures for complete destruction of various organisms:

Culture	0.25-sec. hold	0.5 sec. hold
	——(°F.)——	
Escherichia coli	174.5	172.3
		171.7
Staphylococcus pyogenes	162.6	158.9
Shigella paradysenteriae	162.8	162.0
Salmonella typhosa	165.1	162.8
Corynebacterium diphtheriae	161.4	157.4

These studies are of great value, as they show the minimum times and temperatures needed for the complete destruction of the common pathogens. Further work is needed for this type of information on the tuberculosis organism; however, studies with organisms more resistant than *Mycobacterium tuberculosis* have shown that they also will be destroyed at the higher temperatures.

This information has been presented to indicate that the new high heat and vacuum treatments afford a wide margin of safety, bacteriologically. These temperatures should result in complete destruction of pathogens and reduction of the normal flora of the raw milk to the extent that the keeping quality of the finished product would be increased greatly. The problem of post-processing contaminants is far greater than the problem of bacterial destruction by the process itself.

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OUR INDUSTRY TODAY

EFFECT OF GROWTH STAGE, CHEMICAL COMPOSITION, AND PHYSICAL PROPERTIES UPON THE NUTRITIVE VALUE OF FORAGES[1]

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The main purpose served by forages in the diet of dairy cattle is the provision of energy. Assuming that other dietary conditions are adequate, the following scientific axioms prevail:

(a) Size of animal response (milk yield, tissue maintained and gained) = energy intake,

(b) Energy intake = D. M. intake × energy concentration, and, therefore,

(c) Forage energy intake = intake of forage D. M. × concentration of energy in forage.

Since the characteristics of forage which influence the extent to which it is consumed and its energy value per unit of weight represent the common denominators of forage quality, some of the effects of cutting date and/or growth stage upon intake and energy concentration will be discussed here. The basis of this discussion are some data obtained during the past 11 yr. but which have not been published in detail previously.

INFLUENCE OF CUTTING TIME UPON FORAGE INTAKE

A study of the results of feeding trials conducted in the Northeast suggests that dairy cows fed concentrates at the rate of 1 lb. per 3–4 lb. of milk produced, consume considerably more of first-growth forage cut early than of that cut late. For example, forages cut during early June were consumed at the daily rate of 2.5–3.0 lb. of hay equivalent per 100 lb. of body weight; whereas, the intake of those harvested during mid-July was only 1.1–1.7 lb. At these times of the season in the Northeast, the growth stages of first-growth timothy are classified generally as vegetative and late-bloom, respectively. Although these figures suggest that the time of cutting is a dominant factor determining the rate of voluntary consumption of first cuttings, the considerable range of intake for a given cutting date indicates that conditions other than cutting time are involved, and that systematic study is needed to work out means of predicting this characteristic.

Aftermath forages generally are consumed about as well as first-growth forages harvested early. However, at the present time, there are not enough data to provide bases for predicting the intake of aftermath forages.

INFLUENCE OF CUTTING TIME UPON ENERGY VALUE OF FORAGE

In North America, the criteria of the energy value of feedstuffs most widely used are: digestible dry matter (DDM), particularly for pasture crops; total digestible nutrients (TDN), and digestible energy (DE). These criteria are highly correlated; one is readily predicted from either of the other two and they represent the same percentage of the matter or energy consumed. In only rare instances, such as the occurrence of an unusual fat or mineral concentration, is one criterion superior to the others from the standpoint of predicting the animal response. Conventionally, TDN is the basis of feed evaluation and ration formulation used, and it is the criterion for which most data exist.

But, in the instance of forages, what is the nature of the TDN values recorded in tables of feed composition, and to what extent can these values be used in the practical formulation of rations? Some partial answers to this question are suggested by the data in Table 1,

TABLE 1

Representative TDN values (dry basis) of hays as recorded in feed composition tables[a]

Alfalfa Growth stage	TDN	Timothy Growth stage	TDN
	(%)		(%)
Pre-bloom	58	Pre-bloom	63
1/10- to 1/2-bloom	57	Early-bloom	58
3/4- to full-bloom	56	Full-bloom	57
Post-bloom	53	Late-bloom to early seed	54
All analysis	56	All analysis	55

[a] Data taken from Morrison (5).

[1] Presented at the joint meeting of the American Grassland Council and the American Dairy Science Association at North Carolina State College, Raleigh, June 1958.

taken from the Morrison tables (5). Despite the great difference in growth stages represented, the TDN values of alfalfa and timothy hays in the pre-bloom stage were only 9 and 17% higher, respectively, than those of hays of the same species cut at post-bloom stages. As farmers recognize, and as has been demonstrated experimentally, the difference in the response of animals to different lots of forage of the same species can be much greater than the range in these TDN values (Table 1) would indicate. The results of one experiment demonstrating this are shown in Table 2. The hay

TABLE 2

Response of cows to forages cut at different times from same source

Cutting date	Response of cows		Hay	
	FCM	Body weight change	TDN	Intake[a]
	(lb/day)		*(%)*	*(lb/day)*
June 11	42.7	+0.4	66	28.1
July 9	30.5	−0.1	51	21.8

[a] Corrected to 90% dry basis.

prepared from forage cut on June 11 was about 30% higher in TDN and was consumed at a rate about 30% greater than that of the hay harvested on July 9. About 40% more milk was produced and 0.5 lb. more of body weight was gained per day by the cows consuming the early-cut hay than by those fed the late-cut hay. If the body weight changes represent body tissue (rather than gastrointestinal fill), it could be argued that the net response was about 60% greater for the June 11 hay than for the July 9 hay. Thus, it is clear that forages, even of the same species, may be greatly different with respect to the response they elicit, but that the usual data recorded in tables of feed values do not reflect the magnitude of these differences.

The relatively small degree of variation in the representative TDN values shown in Table 1 is probably the result of the following conditions: *(a)* The data are averages and the classification of the growth stage is based upon the opinions of many people, *(b)* the conventional growth-stage categories may include a considerable range in chronological time and may not adequately serve as indicators of the physiological changes in the plant which are associated with the feeding value, and *(c)* the TDN values of first-growth and aftermath forages of the same growth stage are pooled in the data of the usual feed-value table. This tends to restrict the range of TDN values, for growth stage has much less effect upon the TDN value of aftermaths than upon that of first-growth forages. Also, first-growth forages may have the same TDN value as aftermaths, even though cut at a later physiological growth stage. Obviously, some means of predicting the energy value of forage is needed, in order that livestock may be rationed more accurately than the feed-value tables will allow.

Results of Cornell experiments dealing with time of cutting. It was the object of the experiments to be reported here to study the effect of cutting time upon the TDN value of forages and to attempt to quantify this relationship for prediction purposes. Conventional digestion trials were conducted with 94 first-cuttings and 25 aftermath forages, harvested during ten different seasons. Although most of the forages were mixtures of species commonly grown in the Northeast, 20 were grown as pure stands of eight species. In a few instances, late-maturing varieties were compared with earlier maturing ones. Of the first cuttings, 19 were fed as fresh, green forage, 15 as silage (without preservatives), and 60 as hays. The first-growth forages were harvested at various times ranging from May 5 to July 29.

From the data obtained, it was found that the relationship between the time of cutting in days elapsing after April 30 *(X)* until July 12, and the percentage of DDM *(Y)* in first-growth forage, may be predicted by the equation: $Y = 85.0 - 0.48X$. The standard error of estimate was 1.65%. Possibly because of bottom regrowth and seed formation, the DDM value ranged from 50 to 52% from July 12 to July 29. The rate of decline (0.48% per day) in the DDM value is very similar to that found by other workers, or computed from the data reported by others (2, 3, 6, 7, 8). Although no systematic attempt was made to quantify the relationship between the number of days of regrowth of aftermath forage and its DDM value, the DDM value of all aftermaths studied was between 57 and 64%, regardless of the growth stage. Despite the fact that a number of aftermaths were in the vegetative stage of growth, none of these contained as much DDM as did a first cutting harvested before June 10.

These studies indicate that the species of plant has relatively little or no effect upon the energy value of forage. Although the limited studies made of two varieties each of Bird's-foot trefoil and timothy, having considerably different flowering dates, revealed no differences in the DDM value when the forages were harvested at the same time, more detailed studies involving more forage plants need to be made. Raymond (7) has observed, however, that a leafy, late-maturing strain of ryegrass was approximately three to four percentage units higher in DDM than earlier maturing plants of this and other species cut at the same time. Should it be proved eventually that later-maturing, leafier forage species are only slightly more digestible than their earlier-maturing counterparts (as Raymond's data indicate), it would appear that the relationship between the cutting time and DDM value still would represent the best means available for

the prediction of the energy value of first-growth forages.

Since the relationship between the cutting time and the DDM value was not different for forages fed in different forms (*i.e.*, fresh, green forage, silage, and hays), it was concluded that the method of curing neither improves nor deteriorates the energy value, provided that weather damage is not appreciable. Nevertheless, as the data in Table 3 indicate, the method

TABLE 3

Effect of weather damage upon digestible dry matter value of forage

Cutting date	Treatment	Digestible dry matter (%)
June 10	No weathering–heat-dried	67
June 10	Deliberately weathered–field-cured [a]	57
July 8	No weathering–heat-dried	52

[a] Water added, or rain on all except two days of the eight-day curing period.

of curing can prevent the loss of DDM. It is interesting to note that, although the DDM value of forage harvested on June 10 was markedly reduced by deliberate weathering, the value of the weathered forage was still appreciably higher than that of forage from the same source which was allowed to continue growth until July 8, at which time it was harvested and cured with heated air.

The present data do not provide an indication of the extent to which the relationship between the cutting time and digestibility can be applied to different geographic areas. Further study is needed to determine what affects the *X* intercept and by how much. An examination of the data obtained in the present studies and in the experiments of others revealed that the rate of decline (*i.e.*, the regression coefficient) of the DDM value is essentially the same for forages grown in northeastern United States, England (7), Norway (2), and Sweden (3), but that the *X* intercept values are decidedly different. This infers that climatic conditions might influence the *X* intercept value. The intercept values obtained in the Cornell and English experiments are similar, despite the fact that the latitudes at which the experiments were conducted are approximately 42 and 52 degrees North, respectively. It seems possible that forage grown in England benefits from, *i.e.*, is warmed by, the Gulf Stream; whereas, that grown in northeastern United States might be somewhat chilled by the Labrado Current. Based upon these studies, it would appear that the forage-growing season in Norway (Lat., *ca.* 60° N.) and Sweden (Lat., *ca.* 61° N.) is approximately 19 to 20 days later than that in England and the northeastern region of the United States. The relationship between the cutting date and digestibility of forage discussed above probably would not apply to the forages grown in the southern region of the United States, or to those grown in other areas in which forage does not experience a dormancy during the winter months. Although the basic reasons are not known as to why the rate of decline in the DDM value of forages is similarly related to time, regardless of the country in which it was grown, and the time of the forage-growing season is so greatly different for the four countries, it seems possible that such conditions as light, soil temperature, and the extent of root reserves might be the underlying forces. With the hope that the relationship involving cutting time might be applied more generally in practical feeding, experiments have been begun at Cornell to study the nutritive value of forages grown in various climatic regions of New York State.

The findings of the present studies, as well as those of numerous experiments conducted previously, demonstrate the superiority of early-cut forages over those harvested late. Also, they imply that at the present time the first cutting is usually made too late to avail the farmer the economic benefits of high-quality forage. One of the reasons, other than poor curing weather, why the extension worker and others encounter some reluctance of the farmer to harvest forage earlier than it is now being harvested is the smaller dry matter yield which is associated with early cutting. For example, the data obtained by MacDonald (4) suggest that at Ithaca, New York, forage dry matter yields expressed as a percentage of the maximum first-growth yield (which is generally reached by July 1) are approximately as follows at various dates: May 15, 50; June 1, 73; June 15, 89; June 30, 98, and July 15, 99. However, the dry matter yield per se is a poor index of productiveness. Thus, even though the dry matter yield on June 1 is only 73% of the maximum yield, on this date the energy yield in terms of DDM is approximately 91% of the maximum energy yield, which is generally reached between June 10 and June 21 in the Northeast. Further arguments for moving the cutting date towards June 1 are: *(a)* Animals will consume more of the early-cut forage than of that harvested later; thus, the amount of concentrates needed is less, and *(b)* the apparent sacrifice of energy on a per-acre basis caused by the earlier cutting generally will be more than compensated for by the yield of the second cutting. Thus, cutting the first growth early makes it possible to utilize soil moisture, which usually is abundant during the early part of the season, to promote the growth of the aftermath.

Dry matter content of forage as a simple indicator of nutritive quality. In the search for simple indices of forage quality, the dry matter content at harvesting time has not received

much attention. In a study of 28 forages, representing a variety of species ranging in dry matter content from 15 to 40%, it was observed that the dry matter content *(X)* and digestibility *(Y)* were correlated to the extent of 0.8 and the equation, $Y = 87.4 - 1.042\,X$, relating these two variables, has a standard error of estimate of 4.18%. Aftermath forages appeared to conform equally as well as first-growth forages. Since these data were obtained in experiments not designed to study this particular relationship, and a considerable proportion of the variation observed is probably the result of surface moisture (since no precautions were taken to control it), further study of this relationship under carefully controlled conditions might provide evidence for an excellent, simple index of forage quality which would apply to first-growth and aftermath forages equally well.

Leaf content of forage as an indicator of nutritive quality. The nutritive qualities of the leafy portion are known to be superior to the stems of plants; therefore, it is axiomatic that the proportion of forages comprised by leaves reflects nutritive quality. Experiments at Cornell were conducted with 15 first cuttings and seven second cuttings of several pure species of both legumes and grasses and mixtures of forages, in an attempt to relate the leaf content *(X)* with the DDM value *(Y)*. It was found that the relationship between these variables for first-growth forage is different from that for aftermaths. Aftermath forages ranging in leaf content from 55 to 92% were not much different in DDM value. On the other hand, the leaf content and DDM value of first-growth forage were highly correlated ($r = .95$) and their relationship was expressed by the equation, $Y = 0.4\,X + 40.8$. The first-growth forages examined ranged from 32 to 87% leaves. (In legumes, the leaf was considered to consist of the leaf and petiole; in grasses, the leaf consisted of the blade and sheath.)

Prediction of digestible protein from total protein content of forages. In North America, protein is rationed to ruminants on the basis of apparently digestible protein. In experiments conducted at Cornell, it was found that both the amount and digestibility of protein in forages decline as growth approaches the mature stage. Also, the variation effected by growth stage within a species of plant was found to be as great as that among species. Thus, the average values for the content and digestibility of protein, being categorized chiefly on the basis of botanical composition, in tables of feed values, can be quite erroneous.

As a consequence, a study was made of the relationship between the concentrations of total protein *(X)* and apparently digestible protein *(Y)* in forages. The total protein content of the 79 forages studied ranged from 4 to 28%. Included in the population of forages were only green and dried forages for ensiled forages, and those containing grain were found not to conform. It was found that these variables are highly correlated ($r = .99$) and that the percentage of apparently digestible protein can be predicted from that of total protein by use of the equation, $Y = 0.929\,X - 3.48$. The standard error of estimate is only 0.46%. A similar study (1) made of the data recorded in the Morrison tables (5) resulted in essentially the same equation. Thus, the use of this equation allows the apparently digestible protein content of forages to be predicted accurately from the concentration of total protein, and obviates its actual measurement.

The conditions resulting in the above-recorded relationship appear to involve chemical compositional relationships: *(a)* As maturity is approached, the concentration of fiber increases and that of protein decreases in the forage and *(b)* concomitantly, the proportion of the total protein in the feces comprised of metabolic fecal protein gradually increases. Therefore, since the metabolic contribution to the protein of the feces is high when the forage contains a low concentration of protein (and vice versa), the apparent digestibility of protein in forage containing a low concentration is low (and vice versa). This suggests further that the true digestibility of protein in forages is approximately the same (and is probably of the order of 80 to 90%), regardless of the concentration of protein in the forage.

SUMMARY

The date at which first-growth forage is harvested is the major known determinant of the intake and digestibility of forage by ruminants. At least within a given climatic region, the digestible energy (or matter) value of first-grown forage can be predicted quite accurately. Species of plant, method of preservation (provided that leaf loss is not disproportionate), and physiological growth stage have very little or no effect upon the relationship between cutting time and the DDM value of forage. As growth approaches maturity, the rates of decline in the DDM value of first-growth forages grown in the northeastern region of the United States, and in England, Norway, and Sweden are found to be almost identical.

As growth approaches maturity, the DDM value of aftermath forages declines much more slowly than that of first-growth forage. Regardless of its stage of growth, aftermath forage has a lower DDM value than first-growth forage harvested prior to June 10 in the northeastern states. High rates of nitrogen fertilization and irrigation did not affect the DDM value of aftermath forages.

As a simple index of quality, the dry matter content of forage at the time of harvesting was found to be superior to the leaf content. First-growth and aftermath forages conform to the same relationship between dry matter content and DDM value, whereas the relationship be-

tween the leaf content and DDM value of first-growth forage is different from that of aftermath forage.

Both the amount and digestibility of protein in forages decline as growth approaches maturity. The percentage of apparently digestible protein can be predicted acurately from the concentration of protein in both first-growth and aftermath forages, thus obviating the need for its actual measurement.

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CONDITIONS INFLUENCING FORAGE ACCEPTABILITY AND RATE OF INTAKE[1]

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Total intake of forage is the beginning point in forage evaluation, since it represents the initial quantitative measure of available feed. In attempting to describe this area of interest, certain terms have been used to describe broad areas of influence—the most frequently used terms being palatability, satiation, bulk, and dry matter consumption. The primary problem in determining the individual effects of these factors on forage intake is the inability to assign numerical values to nonspecific or indefinable terms. In a general way, we can define the desired level of intake as that which is sufficient to sustain the desired level of animal production. We can also describe the general problem by pointing out that the animal has a predetermined capacity for forage and that any factor which slows the rate of movement of material through the digestive tract, or results in less than maximum use of this capacity, is reflected in a lowered intake of forage. It is the purpose of this paper to consider some of the factors which aid or prevent the animal in its efforts to consume the desired amount of forage. To do this we shall consider three separate items—the animal, the forage, and the herd and forage management.

The animal. The cow contributes at least three sources of variation to intake figures for a given forage—*(1)* individuality, *(2)* level of production, and *(3)* body size.

The effect of body size is easily handled by correcting intake data for body weight, since this relationship is usually high. Mac Lusky (7) found an over-all correlation of .980, Legates (6) a correlation of .390 with hay, and McCullough (9) a correlation of .897 with individually fed dairy heifers on alfalfa hay.

The effect of level of production on dry matter intake has been studied by Jarl (3), Wallace (13), McCullough (8), and Cox (1). Cox found a correlation of .660 between level of milk production and intake, and McCullough a multiple correlation of .638 between intake, level of production, and dry matter digestibility. In Table 1, the calculated intakes of forage at different levels of milk production are shown. It is of interest to note that the general effects of milk production on forage intake are quite similar, although different forages and management conditions were used by the three stations.

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TABLE 1
Calculated differences in TDN intake due to level of milk production

Level of milk production	TDN consumed per day		
	Jarl, Sweden	Wallace, New Zealand	McCullough, Georgia (U.S.)
15	17.5	17.6	16.0
20	18.2	19.4	17.0
25	18.9	21.1	18.0
30	19.6	22.9	19.0

This increase in dry matter consumption with increasing production is not easily demonstrated. The high coefficients of variability between cows frequently result in average intakes which show no relationship between intake and milk production.

The individuality of animals adds factors which are difficult to measure and evaluate. Mac Lusky (7) found an average coefficient of variation in intake of 21% with free-grazing cows. Kruger (5) found that the feed capacity of mature cows varied from 122 to 196 satiety units, or from 30 to 49 lb. of green corn per cow. This difference in intake partially reflects small differences in size and differences in production, but primarily reflects inheritant differences between cows of similar size and production. These differences are not clearly understood, but are frequently large enough to prevent measurable differences in intake, due to other factors, from being apparent.

A further influence on intake is stage of lactation in dairy cows. Kruger (5) in Germany, Legates in North Carolina (6), and Wallace (13) in New Zealand all have shown that the cow does not consume its maximum dry matter intake during the early weeks of lactation, but reaches a plateau at 6 to 10 wk. after freshening.

The forage. The general relationship between stage of maturity and forage intake should be a well-understood factor by all grasslanders. The literature contains numerous references which need not be reported here.

In general, forage intake is influenced by forage quality and is reflected by a positive correlation (.512) between dry matter digestibility and dry matter intake [McCullough (8)]. This relationship reflects the greater rate of passage and the resulting increase in intake on high-quality forage. This factor is probably one of the items measured when intake is correlated with crude protein or crude fiber.

In addition to stage of maturity, the forage influences intake through such items as quantity available per acre, density per square foot, mixture of species, and palatability.

In Table 2 are shown data relative to the influence of dry matter per acre on average daily intake of forage. The data appear to be contradictory and to indicate only a general relationship. The Holland data, Van Der Kley (12), show an optimum intake at 2,500 lb. of dry matter per acre, whereas the data of Johnstone-Wallace (4) have an optimum intake at about 1,000 lb. dry matter per acre. This apparent difference may be explained in part by the finding of Cox (1), which showed a highly significant relationship between density of forage and dry matter intake. Animals apparently exhibit higher intakes on a dense sward which is about 4 to 6 in. in height, and reduce their consumption as the forage grows taller; or on sparse forage, even if it is optimum in height.

A mixture of forages may influence intake by encouraging excessive selective grazing. Springfield (11) found a positive correlation of .69 between moisture content of the species and the per cent of the species in the diet. A striking example of the effect of species on grazing behavior in dairy cows is shown in Table 3. These data illustrate the effect of changing cows from an unpalatable forage during the day to a highly palatable forage at night. Although the data do not define palatability, they illustrate the gross effect that it may have on the cow's grazing habits.

TABLE 2
Influence of quantity of forage per acre on dry matter intake

Holland (12)		United States (4)	
Dry matter per acre	Dry matter intake	Dry matter per acre	Dry matter intake
(lb.)			
2,500	44.0	1,200	20
1,568	33.0	1,000	32
672	15.4	500	20
		250	10

The longer grazing time on the fescue clover lots does not denote a greater intake of forage. Increases in grazing time reflect short forage, mixed swards, or lower quality forage. The decrease in grazing time by the animals on the oat forage probably reflects a greater intake of dry matter per unit of grazing time.

TABLE 3
Effect of grazing management on total and the division of grazing time (Georgia)

Lot No.	Per cent of		Per cent of grazing time		Total grazing time
	Clover	Fescue	Day	Night	
2	7	82	47	43	450
3	21	69	43	57	465
On Lot 2 daytime and on oats at night			36	64	435
On Lot 3 daytime and on oats at night			29	71	352

TABLE 4
General effect of forage digestibility on rate of dry matter intake

Forage	Forage (dry basis) Consumption cow/day	Consumption cow/hr grazing	Digestibility
	(lb.)		*(%)*
Bermuda grass	13.5	1.67	59
Starr millet	19.6	3.13	73
Oats, ryegrass, crimson clover	22.1	2.90	70

This general relationship is shown in Table 4 (Georgia data).

Management. Under the heading of management are included those man-inflicted practices which alter plant and animal response. Such practices are initiated under the title of grassland or herd management, and represent the efforts of a farm manager to properly manage his feed and animals to obtain a desired level of forage intake. The practices include strip grazing, limiting time on pasture, and supplemental feeding. The results of several such practices are shown in Table 5.

The first group of data illustrates efforts to decrease forage consumption on winter oats at the Georgia station. Following the observation that cows spent about 8 hr. per day grazing under these conditions, efforts were made to limit forage intake by reducing grazing time to 6 or 3 hr. Hay was fed free-choice when the cows were not on pasture. The cows that grazed for two 3-hr. periods consumed more forage than those that grazed continuously. When the cows were grazed after each milking but not at night they also consumed more forage than those grazed continuously.

The second group of data are those reported by Seath (10), and Dowden (2), at the Kentucky station, and represent the results of their efforts to supplement permanent pastures during the summer. The feeding of grain, or grain and hay, or silage was essentially without effect on increasing total dry matter intake.

The third group of data represents the results of supplementing two silages at the Georgia station. When Sudan silage with a TDN content of 71% was supplemented with hay it resulted in a lower total dry matter intake. Oat silage, with a TDN of 60%, was supplemented with hay and grain and this procedure resulted in an increase in intake. This variation in the effect of supplemental feeding was also observed by Mac Lusky (7) in grazing trials. The data are summarized in Table 6.

TABLE 6
Effect of supplements on herbage dry matter consumption

Item	Period (dry matter per cow) 1	2	3	4
With 7.2 lb. supplement dry matter	23.3	26.2	13.5	19.1
No supplement	24.1	26.8	17.6	24.5
Difference	0.8	0.6	4.1	5.4

SUMMARY

There are at least three factors which influence forage intake—the forage itself, the animal and its needs, and the conditions imposed by the manager. The animal apparently makes an effort to meet its nutrient needs, but may be limited by mechanical satiation. The influence of palatability is recognized, but no means of measurement has been perfected. With grazing animals, the quantity and qual-

TABLE 5
Results of several attempts to alter intake by management or feeding

Ration	Dry matter intake Supplement	Total
Pasture—Georgia	*(Pasture)*	
Pasture—24 hr.	28.1	..... 28.1
Pasture—2 × 3 hr. grazing periods plus hay	33.6	6.1 39.7
Pasture—1 × 3 hr. grazing period plus hay	14.6	11.4 26.0
Pasture 24 hr.	24.7	..... 24.7
Pasture—7 A.M.–1 P.M. and 4:30–6:30 P.M.	26.9	..... 26.0
Pasture—Kentucky		
Pasture alone	37.5	0 37.5
Pasture plus 6 lb. grain	30.1	6.0 36.1
Pasture plus 12 lb. grain	28.0	12.0 40.0
Pasture plus grain	27.4	3.8 31.2
Pasture plus grain plus hay	18.1	12.6 30.7
Pasture plus grain plus silage	23.8	8.0 21.8
Silage—Georgia	*(Silage)*	
Oat silage plus grain	20.8	6.3 27.1
Oat silage plus hay plus grain	20.9	11.5 32.4
Sudan silage alone	27.7	..... 27.7
Sudan silage plus hay	18.6	7.2 25.8

ity of forage available, as well as the density of that forage, apparently exerts an influence on forage intake. In general terms, the problem of intake can be summarized as follows: At a stated level and stage of animal production, a forage may be defined as adequate when it is supplied in such a quantity, is of sufficient palatability, and its per-unit concentration of usable nutrients is high enough to permit the animal to meet its nutritional requirements concurrent with the satiation by bulk.

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METHODS OF FORAGE UTILIZATION IN THE MIDWEST [1, 2]

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There is little doubt that the cheapest source of nutrients: nitrogen, energy, minerals, and vitamins, for our dairy cattle is forage. Yet we have spent relatively more in time and energy in making and feeding a better concentrate than we have in feeding a better roughage. Our overall picture of summer forage utilization is inefficient but, fortunately, farmers are coming to the realization that their pastures can be as productive as most of their good crop acres.

In the Middle West, grazing cropland acres may become a thing of the past. Dairy farmers are coming slowly but definitely to the realization that pasturing is not the most efficient method of harvesting forage. Farmers are moving the milking herd out of unproductive woody and hilly land, spurred largely by the ever-present and inevitable summer slump. This drop in milk production, which occurs every year, is usually counteracted by supplemental feeding of hay and silage, as well as by supplying better forages, primarily, drought-resistant grasses, grass legume mixes and, in recent years, by going to better methods of pasturing, such as rotation and strip grazing. In addition, two methods of summer feeding eliminating the dairy animal as the harvesting machine—green feeding and stored feeding—are coming into the picture. None of these systems is new, but since our farmers are becoming more forage-conscious and our forage crop is being considered as a cash crop, these better and less wasteful practices are coming into use.

The concept of using the dairy cow as the forage harvester is deeply ingrained in our Midwest dairy operation and, indeed, in some areas of the Midwest dairy area this is the only way the job of summer-forage harvesting can be done. However, in large areas where the cow is harvesting its own feed on expensive cropland acres, waste is obviously exorbitant, selective grazing is excessive, possibility for regrowth is

[1] Presented at the joint meeting of the American Grassland Council and the American Dairy Science Association at Raleigh, North Carolina, June, 1958.

[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

nonexistent, and energy expended by a large grazing herd is extremely wasteful. A statement made by Josiah Quincy of Massachusetts in 1859 seems nearly as applicable today as then, and is here quoted: "A mistaken notion that a considerable extent of land is requisite to enable a farmer to keep many head of cattle led to a wasteful portion of it being retained for the sole purpose of pasturing, and thus compared with its inherent productive power was made useless."

The methods of interest to us today of harvesting our summer forage have been in use in various degrees of intensity for many years, and many of the problems of past years are still plaguing us today. In the Midwest, estimates are that most producers still follow the time-honored method of open grazing, not more than 15% conduct some method of controlled grazing, at the most from 5 to 10% practice green feeding, and those feeding hay and silage year-around, no more than 5%. Soils and agronomic scientists have made great strides in developing species of grasses and legumes in combinations which produce good volumes of high-quality, nutritious, and palatable forage, but we have incorporated very little of what we do know about efficient harvesting of these combinations of summer forage into our summer feeding programs. In addition, we know little concerning these forage combinations in terms of their relative acceptability, the physiological effect on the grazing animal in relation to production, health, degree to which these forages satisfy the needs of the high-producing dairy animal, the efficiency with which it uses the plant nutrients, and the relative grazing habits of the dairy cow on the various species combinations.

In the past few years it has been noted that the milking herds are becoming larger, number of farmers fewer, and the size of farm larger. With these changes is also coming vertical expansion in the realm of dairy production and with this must come more efficient use of our cropland acres. Therefore, as a result, we are witnessing an increase in the number of farms incorporating some of the more efficient ways of utilizing summer forage. Table 1 shows that efficient methods of harvesting must come into use if herd expansion is to take place without a large increase in farm size.

It is the object of this report, practically and briefly, to present data obtained from experiments using alternative systems of summer utilization of forage and where these systems may fit the Midwest dairy operation.

EXPERIMENTAL RESULTS RELATING TO METHODS OF SUMMER FEEDING

A large portion of the work reported in the Midwest has been conducted in determining the differences in return between continuous and some type of rotation-grazing scheme. In progress now are experiments dealing with green feeding and stored feeding. Depending upon the conditions imposed in the experiment, we generally find that the greater the control of the grazing program, the greater the efficiency of utilization of standing forage. This is pointed out in Table 2, from data of work carried out with dairy animals at a number of Midwest stations.

With the limited data available, it seems quite clear that the dairy animal as a harvesting unit is very extravagant. It should be pointed out that the forages dealt with in the work referred to in Table 2 were tall-growing forages of a

TABLE 1

Approximate number of acres required per cow per 110–120-day pasture season

Kind of pasture	Method of grazing	Acres/ cow/ season
Woods pasture	Continuous [a]	10–20
Renovated (open fields)	Continuous	3–5
Grass—Legume mixture	Continuous	2–4
Grass—Legume mixture	Rotation [b]	1–2
Grass—Legume mixture	Strip grazing [c]	¾–1
Grass—Legume mixture	Green feeding [d]	½–1
Grass—Legume mixture	Stored feeding [e]	½

[a] Continuous grazing—no more than two separate grazing areas per season.

[b] Rotation grazing—a new grazing area not oftener than every two days.

[c] Strip grazing—a new grazing area at one-day intervals or less.

[d] Green feeding—green-cut forage harvested and fed in dry lot.

[e] Stored feeding—cured forage fed during the entire pasture season.

TABLE 2

Efficiency of harvesting forage by dairy cattle (3-yr. average)

	D.M. available	D.M. consumed	D.M. uneaten	Per cent loss
	Per acre			
Conventional	4,250	1,275–1,700	2,975–3,550	60–70
Rotation grazing	5,856	3,318	2,538	43
Strip grazing	6,108	4,188	1,920	31
Green feeding [a]	5,469	5,358	111	2
Stored feeding [a]	7,099	6,326	773	11

[a] Values obtained from forage as fed.

predominantly alfalfa and grass mixture. This type of forage when subjected to any kind of a grazing plan is very susceptible to fouling by animal activity. In experiments in other areas and countries where low-growing forages are the predominant pasture herbage, no great difference is noted between a conventional and rotation-grazing plan. The loss of forage when green feeding or stored feeding is governed almost entirely by the quality and quantity as fed, and the amount of waste allowed by the manger or bunk in which it is fed.

The actual return in terms of animal product is the main criterion by which we usually judge our feeding program. However, milk production alone does not reveal the entire picture. We must know and account for the level of grain feeding and supplemental hay feeding which will materially affect the level of production and consumption of pasture forage. Under a well-managed system of summer utilization of forage, we find little if any change in milk production on a per-cow basis, provided sufficient high-quality forage is available. Table 3 shows considerable difference, however, on an acre basis. Data have been corrected for concentrate feeding by the indicated relationship and in every case was fed according to individual animal need.

The data of Table 3 have been obtained over three consecutive years, from an experiment now in progress at two stations in Wisconsin. The experiments have a number of years to run, which may somewhat affect the results. However, the trends noted in relation to increased production per acre toward the more controlled feeding program have been consistent throughout the course of the trials.

In addition to the increased productive capacity in terms of milk production of pasture acreage, being shown in favor of the intensively managed pasture system, this is also demonstrated by the animal-carrying capacity of forage acreage as shown in Table 4.

Many factors affect the carrying capacity of forage acreage. Environmental conditions in terms of rainfall and temperature directly affect the grazing habits of the animals. These conditions also have a direct bearing upon the rate of growth, plant species ratio, and density and height of sward, which are also affected by topography, drainage, fertility, and frequency of grazing or harvest. The attempt is made in the above-mentioned experiments to distribute evenly all factors mentioned over all treatments, with the exception of the latter, which varies with each individual method of forage utilization. The level of supplementary feeding will have considerable effect on carrying capacity, as well. Supplementary hay was fed at low levels to the animals on one experiment dealing with rotation and strip grazing. However, acreage required has been calculated with consideration given to the amount of supplemental hay consumed on the basis of dry matter production of the pastures.

TABLE 4

Carrying capacity of forage-producing land (110- to 120-day season)

System of utilization	Acres per cow per season	Cows per acre per season	Decreased acre requirement over rotation
Rotation	1.14	0.9	
Strip grazing	0.76	1.3	0.38
Green feeding	0.69	1.5	0.45
Stored feeding	0.49	2.0	0.65

PRACTICAL ASPECTS OF SYSTEMS OF FORAGE UTILIZATION IN THE MIDWEST

In contacting the various researchers of the Midwest who have conducted research in the area of systems of summer pasture and forage utilization, we find that there is fairly general agreement that reasonably high levels of milk production can be maintained under any of the systems previously discussed. Management of the pastures or areas harvested must be such that sufficient feed is available at all times to maintain body weight, milk production, and animal health. In the experiments carried on by the University of Wisconsin, this criterion is closely adhered to. Consequently, as indicated by the data presented, the difference in level of milk production between groups is inconsequential, but the land area required to provide the necessary forage is quite different. We know that this is not true of the average farm

TABLE 3

Milk production (4% FCM) under various systems of summer feeding (3-yr. average)

	Milk per cow per milking day	Pounds milk per acre[a]	Actual milk per acre	Milk per acre increase over rotation	Level of concentrate feeding per day
			(lb.)		
Rotation grazing	34.3	2,411	2,757		4.5
Strip grazing	37.1	4,292	5,044	1,881	5.2
Green feeding	37.9	5,190	5,958	2,779	4.9
Stored feeding	36.3	6,056	8,135	3,645	8.3

[a] Per cent of TDN received from forage × total FCM.

set-up since, for the most part, low production is not due primarily to grazing system for summer feeding but mainly to lack of feed in the system. In view of this observation, a general recommendation (trite though it seems) for any summer feeding program would be to make certain that the milking cow receives plenty of high-quality forage. Table 5 presents the pounds of green forage consumed by a 1,300-lb. Holstein cow producing from 30 to 50 lb. of milk, and the approximate area needed to supply that forage.

Stored feeding. From the research work under way at Wisconsin, stored feeding continues to require less acres per cow per season. This requirement per cow has remained surprisingly

TABLE 5

Approximate area and amount of green forage required per 1,300-lb. cow per day

System	Pounds green feed per day	Approximate area (sq. ft.) required per day [a]
Rotational	130 to 150	1,000 to 2,000
Strip grazing	130 to 150	500 to 1,100
Green feeding	140 to 180	300 to 1,000
Stored feeding	50 to 60 [b]	

[a] Depends upon stage of growth, maturity, density, acceptability, and species combination.

[b] Hay and silage.

constant, with little variation in spite of the fact that we have experienced quite wide variations in environmental conditions.

Other advantages for a system of stored feeding have become evident as we have gained experience with this method. The forage can all be harvested at the same time, at near the same stage of growth, with the smallest amount of field losses as our system of harvesting will allow. If we carry on this system for the entire year, we can eliminate most fences, allowing for better land management, greater ease in moving from field to field, and no hindrance in adopting the practices of strip farming on the contour where topography dictates such a practice. The land saved from fence rows and added to the forage-producing land is not inconsequential on dairy farms of today's size.

Stored feeding allows the use of storage capacity year-around, but it demands the incorporation into the forage-harvesting system of the very best accepted practices, in order that good-quality forage be fed the year around. If good-quality forage is fed in amounts to supply the necessary nutrients for optimum milk production, no summer drop in milk production need be experienced. In our experience with this system we have never been able to detect a prolonged measurable increase in milk production due to early spring grazing or green feeding. In keeping the cattle off the forage-producing land, a considerable amount of forage is saved from destruction by the grazing animal. The losses are caused primarily by walking, lying, dunging, urinating upon it, and by frequent and severe cropping, not allowing for sufficient time to recover.

A dry lot must be provided close to the barn and feeding area, which may be a concrete barnyard where the animals are fed, if they are not fed in the winter feeding area. We have found that a good blue grass sod is very advantageous in our stored feeding operation and, except for periods of very adverse conditions, the animals use it almost continuously. With the milking herd near the buildings at all times, close observation of the animals for signs of heat, injuries, and sickness is greatly facilitated. The problem of bloat is reduced considerably. Facilities for watering the herd need little alteration to supply a readily available supply of water for the stored-fed herd.

Inconvenience of such a system may be manifested in a number of ways. Sanitation in the vicinity of the farm buildings may become a distinct problem, due to the high concentration of animals, if regular precautions are not taken. Many farmers look forward to spring, when the cows can be put to pasture, thus freeing them from the regular winter barn work. This system, in many instances, may not allow freedom from the winter routine.

It should be pointed out again that the predominance of work done in the Midwest has been with tall-growing grass-legume mixtures. With low-growing grasses and legumes, the advantages will, by and large, be decreased or nullified. Also, if the topography of an area or a single farming unit is such that harvesting by means other than the animal is not possible, the arguments in favor of stored feeding do not hold.

Green feeding. Many of the advantages for a system of hauling green chopped forage to the milking herd are similar to those described above for stored feeding, namely: *(a)* saves forage by reducing waste from detrimental grazing habits, *(b)* animals are near the buildings where they can be under close surveillance, *(c)* eliminates the necessity of fencing fields, *(d)* no special provisions need be made for water, *(e)* acreage required per cow is considerably less than most grazing systems, *(f)* the problem of bloat is lessened, since the animal can not select portions of the plant but must eat it in its entirety, *(g)* milk flow should be continuous and not fluctuate as severely as on a continuous grazing system, *(h)* no detrimental effects have been noted by being confined to a small area, since a sod lot can be provided for the cattle for an exercise area.

A rule in any summer feeding plan should be to let an animal take such exercise as it chooses to promote health—not to compel it to work many hours a day for a living.

The disadvantages of green feeding appear to be of greater magnitude than those of stored feeding. Stored feeding probably can be prac-

ticed with most any size dairy herd with equal efficiency. However, when considering green feeding as a summer-feeding program the general recommendation is that with herds of less than 30 cows such an operation would be quite inefficient. Other disadvantages would be: *(a)* additional daily labor and time are required. To be conducted successfully the feed should be cut twice daily to circumvent the rapid heating of the forage, to insure maximum consumption by the animal. *(b)* Odd time-requirement. Generally, harvesting the forage twice daily cuts into the regular field work and other regular responsibilities. Also, attention every day of the week is necessary. *(c)* Additional machinery. A tractor and forage harvester should be available in addition to the regular machinery complement, or be taken from the regular field operation whenever needed. *(d)* Sanitation. This problem is of importance equally with the green feeding system or with the stored forage system. *(e)* Wet weather may prohibit harvesting some days. This drawback is one that becomes serious when wet weather prevails, where the forage comes from a new sod or where annuals such as oats, sorghums, Sudan, or corn are being cut and green-fed to the cattle. In the 3 yr. of green-feeding at the Wisconsin station, where alfalfa, brome, and Ladino have been used exclusively, we have lost less than a week in harvesting days.

Green feeding has proved itself a successful operation on a number of our larger dairy farms of the Midwest. This is primarily due to the fact that this system allows for increasing the herd size without the necessity of increasing the land area, and with larger herd size the labor force can be increased.

Grazing systems. Since the concept of harvesting forage with the dairy cow is deeply ingrained in the economy of dairy farming of the Midwest, it becomes necessary to establish a system of grazing that will return to our farmers who graze their forage-producing land by necessity or tradition the maximum economical return per acre. From our limited experience with rotation and strip grazing, and from reports received from other stations in the Midwest using a grass alfalfa mixture, it becomes increasingly evident that the more intensive the grazing system the greater the return per acre. The per cent forage grazed, the milk production per acre, and the carrying capacity per acre increase. However, along with the greater intensity of grazing comes the demand for greater managerial skill. Some of the disadvantages for increasing the intensity of grazing are: *(a)* Additional time is required for moving the fences to operate such a system. This time requirement has been shown to be about 30 min. per day if the animals are given a new area to graze twice daily, as we have been doing at the Wisconsin station. *(b)* Generally, water must be supplied by portable tank, which adds to the expense and time. We feel that water in close proximity to the area being grazed is of utmost importance. *(c)* During wet weather a group of cattle grazing a small area can do considerable damage to a wet sod. *(d)* Close supervision is of utmost importanee to keep the cattle supplied with the right amount of forage at the stage of growth most conducive to maximum consumption. Regardless of the stage of growth or intensity of grazing we have very seldom been able to graze more than 80% of the forage. The best we have been able to do with rotation grazing is about 60%.

Some of the advantages of an intensified system of grazing, as compared to a less intensified system, are as follows: *(a)* more complete harvesting of forage, *(b)* less acreage per cow per season, *(c)* more milk per acre, *(d)* greater allowance for regrowth of the sward, thus reducing death losses of that portion of pasture mix susceptible to damage by close grazing, *(e)* allows opportunity to clip weedy areas, *(f)* gives more control over daily forage consumption throughout the pasture season.

Only a few of the facets of summer forage utilization have been touched on here. Much of the work referred to is only in its beginning stages, and must await continued experimentation before more conclusive answers will be forthcoming.

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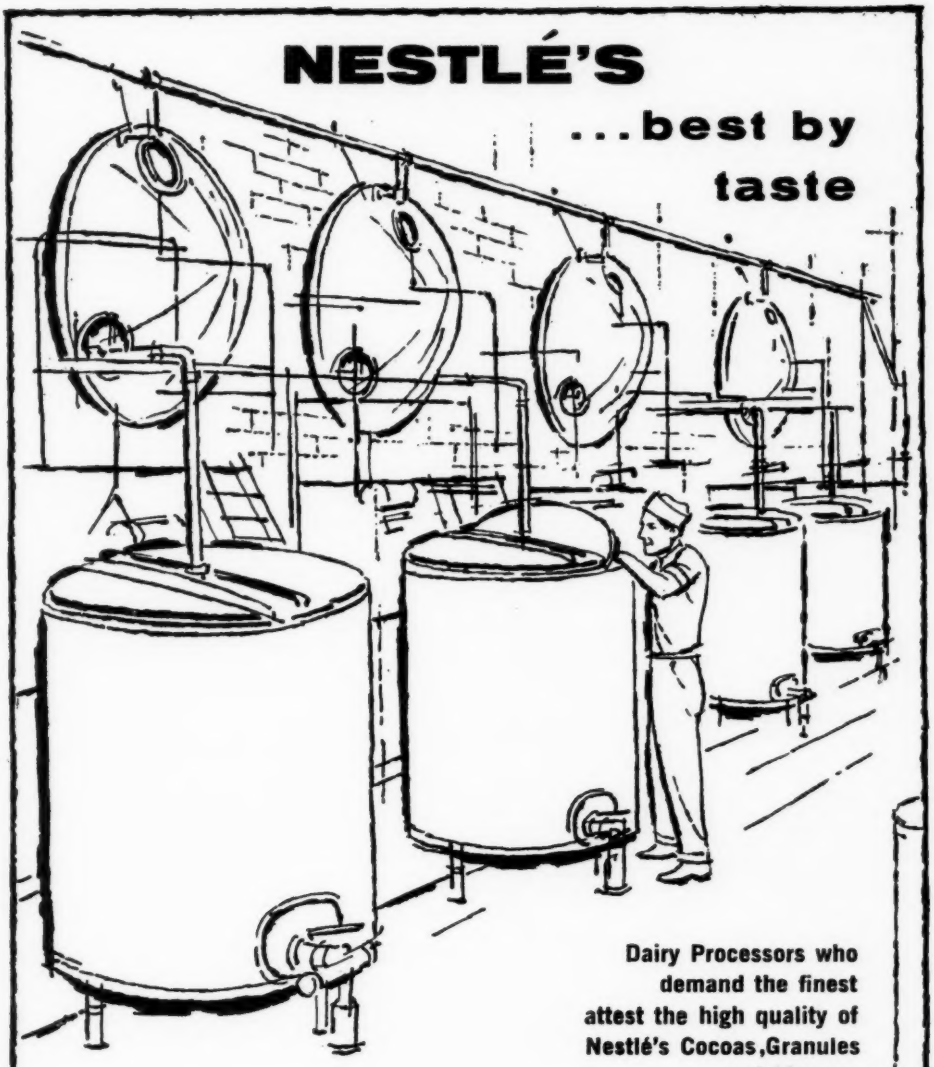

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